

## TUMOR NECROSIS FACTOR RECEPTORS 6 $\alpha$ & 6 $\beta$

*Sub B1*

This application is a continuation-in-part of application serial No. 09/006,352, filed 13 January, 1998, which is herein incorporated by reference in its entirety, priority to which is hereby claimed under 35 U.S.C. §120. In addition, priority of the following provisional applications is hereby claimed under 35 U.S.C. §119(e): Serial No. 60/121,774, filed 04 March 1999, Serial No. 60/124,092, filed 12 March 1999, Serial No. 60/131,279, filed 27 April 1999, Serial No. 60/131,964, filed 30 April 1999, Serial No. 60/146,371, filed 02 August 1999, and Serial No. 60/168,235, filed 01 December 1999, each of which is hereby incorporated by reference in its entirety.

### *Field of the Invention*

The present invention relates to novel human genes encoding polypeptides which are members of the TNF receptor family. More specifically, isolated nucleic acid molecules are provided encoding human polypeptides named tumor necrosis factor receptor-6 $\alpha$  & -6 $\beta$  hereinafter sometimes referred to as “TNFR-6 $\alpha$ , & TNFR-6 $\beta$ ” or generically as “TNFR polypeptides”. TNFR polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of TNFR polypeptide activity. Also provided are diagnostic and therapeutic methods utilizing such compositions.

### *Background of the Invention*

Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intra-cellular response.

For example, tumor necrosis factors (TNF) alpha and beta are cytokines which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

Among the ligands there are included TNF- $\alpha$ , lymphotoxin- $\alpha$  (LT- $\alpha$ , also known as TNF- $\beta$ ), LT- $\beta$  (found in complex heterotrimer LT- $\alpha$ 2- $\beta$ ), FasL, CD40L, CD27L, CD30L, 4-IBBL, OX40L and nerve growth factor (NGF). The superfamily of TNF receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 4-IBB, OX40, low affinity p75 and NGF-receptor (Meager, A., *Biologicals*, 22:291-295 (1994)).

Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., *supra*).

Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., *et al.*, *Nature* 356:314 (1992)), perhaps reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (Allen, R.C. *et al.*,

*Science* 259:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by faulty sensory innovation of peripheral structures (Lee, K.F. *et al.*, *Cell* 69:737 (1992)).

TNF and LT- $\alpha$  are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT- $\alpha$ , acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT- $\alpha$  are involved in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von Huffel, C., *Science* 264:667-668 (1994)). Mutations in the p55 Receptor cause increased susceptibility to microbial infection.

Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is responsible for transducing signals for programmed cell death (Tartaglia *et al.*, *Cell* 74:845 (1993)). Apoptosis, or programmed cell death, is a physiologic process essential to the normal development and homeostasis of multicellular organisms (H. Steller, *Science* 267, 1445-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, *Science* 267, 1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland, J.N. Ihle, *Cell* 81, 479-482 (1995); A. Fraser, G. Evan, *Cell* 85, 781-784 (1996); S. Nagata, P. Golstein, *Science* 267, 1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40,

and CD30, among others (C.A. Smith, *et al.*, *Science* 248, 1019-23 (1990); M. Tewari, V.M. Dixit, in Modular Texts in Molecular and Cell Biology M. Purton, Hedin, Carl, Ed. (Chapman and Hall, London, 1995). While family members are defined by the presence of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain", which is distantly related to the Drosophila suicide gene, reaper (P. Golstein, D. Marguet, V. Depraetere, *Cell* 81, 185-6 (1995); K. White *et al.*, *Science* 264, 677-83 (1994)). This shared death domain suggests that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan, K. O'Rourke, M. Tewari, V. M. Dixit, *Cell* 81, 505-12 (1995); M. P. Boldin, *et al.*, *J. Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio *et al.*, *Cell* 85, 817-827 (1996); M.P. Boldin, T.M. Goncharov, Y.V. Goltsev, D. Wallach, *Cell* 85, 803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF-kB (L.A. Tartaglia, D.V. Goeddel, *Immunol Today* 13, 151-3 (1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu, J. Xiong, D.V. Goeddel, *Cell* 81, 495-504 (1995); H. Hsu, H.-B. Shu, M.-P. Pan, D.V. Goeddel, *Cell* 84, 299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF-kB activation (H. Hsu, H.-B. Shu, M.-P. Pan, D.V. Goeddel, *Cell* 84, 299-308 (1996); H. Hsu, J. Huang, H.-B. Shu, V. Baichwal, D.V. Goeddel, *Immunity* 4, 387-396 (1996)).

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states. In particular, there is a need to isolate and characterize novel members of the TNF receptor family.

### ***Summary of the Invention***

The present invention provides isolated nucleic acid molecules comprising, or alternatively consisting of, a polynucleotide encoding at least a portion of a TNFR (i.e., TNFR-6 $\alpha$  or TNFR-6 $\beta$  polypeptide) having the complete amino acid sequences shown in SEQ ID NOS:2 and 4, respectively, or the complete amino acid sequence encoded by a cDNA clone deposited as plasmid DNA as ATCC Deposit Number 97810 and 97809, respectively. The nucleotide sequence determined by sequencing the deposited TNFR-6 alpha and TNFR-6 beta clones, which are shown in Figures 1 and 2A-B (SEQ ID NOS:1 and 3, respectively), contain open reading frames encoding complete polypeptides of 300 and 170 amino acid residues, respectively, including an initiation codon encoding an N-terminal methionine at nucleotide positions 25-27 and 73-75 in SEQ ID NOS: 1 and 3, respectively.

The TNFR proteins of the present invention share sequence homology with other TNF receptors. Splice variants TNFR-6 alpha and TNFR-6 beta show the highest degree of sequence homology with the translation products of the human mRNAs for TNFR-I and -II (Figure 3) (SEQ ID NOS:5 and 6, respectively) also including multiple conserved cysteine rich domains.

The TNFR-6 alpha and TNFR-6 beta polypeptides have predicted leader sequences of 30 amino acids each; and the amino acid sequence of the predicted mature TNFR-6 alpha and TNFR-6 beta polypeptides are also

2A.3

shown in Figures 1 and 2 as amino acid residues 31-300 (SEQ ID NO:2) and 31-170 (SEQ ID NO:4), respectively.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding a TNFR polypeptide having the complete amino acid sequence in SEQ ID NO:2 or 4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; (b) a nucleotide sequence encoding a mature TNFR polypeptide having the amino acid sequence at positions 31-300 in SEQ ID NO:2, or 31-170 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; (c) a nucleotide sequence encoding a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in the ATCC Deposit No. 97810 or 97809; (d) a nucleotide sequence encoding a fragment of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in the ATCC Deposit No. 97810 or 97809 wherein said fragment has TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  functional activity; and (e) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), or (d) above.

Further embodiments of the invention include isolated nucleic acid molecules that comprise, or alternatively consist of, a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 80%, 85%, 90%, 92%, or 95%, 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences in (a), (b), (c), (d) and (e) above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide in (a), (b), (c), (d), or (e) above. This polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide

having a nucleotide sequence consisting of only A residues or of only T residues. An additional nucleic acid embodiment of the invention relates to an isolated nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide having an amino acid sequence in (a), (b), (c), or (d) above.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of TNFR polypeptides or peptides by recombinant techniques.

The invention further provides an isolated TNFR polypeptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of a full-length TNFR polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 or 4 or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; (b) the amino acid sequence of a mature TNFR polypeptide having the amino acid sequence at positions 31-300 in SEQ ID NO:2, or 31-170 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; (c) the amino acid sequence of a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; or (d) the amino acid sequence of a fragment of the TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809, wherein said fragment has has TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  functional activity.

The polypeptides of the present invention also include polypeptides having an amino acid sequence at least 80% identical, more preferably at least 85% identical, and still more preferably 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to those described in (a), (b), (c) or (d) above, as well as 5 polypeptides having an amino acid sequence with at least 90% similarity, and more preferably at least 80%, 85%, 90%, 92%, or 95% similarity, to those above.

An additional embodiment of this aspect of the invention relates to a peptide or polypeptide which comprises the amino acid sequence of an 10 epitope-bearing portion of a TNFR polypeptide having an amino acid sequence described in (a), (b), (c) or (d), above. Peptides or polypeptides having the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide of the invention include portions of such polypeptides with at least six or seven, preferably at least nine, and more preferably at least about 15 30 amino acids to about 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire amino acid sequence of a polypeptide of the invention described above also are included in the invention.

In another embodiment, the invention provides an isolated antibody 20 that binds specifically to a TNFR polypeptide having an amino acid sequence described in (a), (b), (c) or (d) above. The invention further provides methods for isolating antibodies that bind specifically to a TNFR polypeptide having an amino acid sequence as described herein. Such antibodies are useful diagnostically or therapeutically as described below.

Tumor Necrosis Factor (TNF) family ligands are known to be among 25 the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. The invention also provides for pharmaceutical compositions comprising TNFR polypeptides, particularly

human TNFR polypeptides, which may be employed, for instance, to treat infectious disease including HIV infection, endotoxic shock, cancer, autoimmune diseases, graft vs. host disease, acute graft rejection, chronic graft rejection, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury (e.g., ischemic cardiac injury), toxin-induced liver disease, septic shock, cachexia and anorexia. Methods of treating individuals in need of TNFR polypeptides are also provided.

The invention further provides compositions comprising a TNFR polynucleotide or a TNFR polypeptide for administration to cells *in vitro*, to cells *ex vivo* and to cells *in vivo*, or to a multicellular organism. In certain particularly preferred embodiments of this aspect of the invention, the compositions comprise a TNFR polynucleotide for expression of a TNFR polypeptide in a host organism for treatment of disease. Particularly preferred in this regard is expression in a human patient for treatment of a dysfunction associated with aberrant endogenous activity of a TNFR polypeptide.

In another aspect, a screening assay for agonists and antagonists is provided which involves determining the effect a candidate compound has on TNFR polypeptide binding to a TNF-family ligand. In particular, the method involves contacting the TNF-family ligand with a TNFR polypeptide and a candidate compound and determining whether TNFR polypeptide binding to the TNF-family ligand is increased or decreased due to the presence of the candidate compound. In this assay, an increase in binding of a TNFR polypeptide over the standard binding indicates that the candidate compound is an agonist of TNFR polypeptide binding activity and a decrease in TNFR polypeptide binding compared to the standard indicates that the compound is an antagonist of TNFR polypeptide binding activity.

TNFR-6 alpha and TNFR-6 beta are expressed in endothelial cells, keratinocytes, normal prostate and prostate tumor tissue. For a number of disorders of these tissues or cells, particularly of the immune system,

significantly higher or lower levels of TNFR gene expression may be detected in certain tissues (e.g., cancerous tissues) or bodily fluids (e.g., serum, plasma, urine, synovial fluid or spinal fluid) taken from an individual having such a disorder, relative to a "standard" TNFR gene expression level, i.e., the TNFR expression level in healthy tissue from an individual not having the immune system disorder. Thus, the invention provides a diagnostic method useful during diagnosis of such a disorder, which involves: (a) assaying TNFR gene expression level in cells or body fluid of an individual; (b) comparing the TNFR gene expression level with a standard TNFR gene expression level, whereby an increase or decrease in the assayed TNFR gene expression level compared to the standard expression level is indicative of disorder in the immune system.

An additional aspect of the invention is related to a method for treating an individual in need of an increased level of TNFR polypeptide activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of an isolated TNFR polypeptide of the invention or an agonist thereof.

A still further aspect of the invention is related to a method for treating an individual in need of a decreased level of TNFR polypeptide activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of a TNFR antagonist. Preferred antagonists for use in the present invention are TNFR-specific antibodies.

### ***Brief Description of the Figures***

Figure 1 shows the nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of TNFR-6 $\alpha$ . The initial 30 amino acids (underlined) are the putative leader sequence.

*Figures 2A-3*

*Figure 2* shows the nucleotide sequence (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) of TNFR-6 $\beta$ . The initial 30 amino acids (underlined) are the putative leader sequence.

*Figures 3A-D*

*Figure 3* shows an alignment created by the Clustal method using the Megalign program in the DNASTAR suite comparing the amino acid sequences of TNFR-6 $\alpha$  ("TNFR-6 alpha" (SEQ ID NO:2)), and TNFR-6 $\beta$  ("TNFR-6beta" (SEQ ID NO:4)) with other TNF receptors, as follows: TNFR1 (SEQ ID NO:5); TNFR2 (SEQ ID NO:6); NGFR (SEQ ID NO:7); LTbR (SEQ ID NO:8); FAS (SEQ ID NO:9); CD27 (SEQ ID NO:10); CD30 (SEQ ID NO:11); CD40 (SEQ ID NO:12); 4-1BB (SEQ ID NO:13); OX40 (SEQ ID NO:14); VC22 (SEQ ID NO:15); and CRMB (SEQ ID NO:16).

Figures 4 and 5 show separate analyses of the TNFR-6 alpha and TNFR-6 beta amino acid sequences, respectively. Alpha, beta, turn and coil regions; hydrophilicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown, as predicted for the amino acid sequence of SEQ ID NO:2 and SEQ ID NO:4, respectively, using the default parameters of the recited computer programs. In the "Antigenic Index - Jameson-Wolf" graph, which indicates the location of the highly antigenic regions of TNFR-6 $\alpha$  and TNFR-6 $\beta$ , i.e., regions from which epitope-bearing peptides of the invention may be obtained. Antigenic regions of TNFR-6 $\alpha$ , include from about Ala-31 to about Thr-46, from about Phe-57 to about Thr-117, from about Cys-132 to about Thr-175, from about Gly-185 to about Thr-194, from about Val-205 to about Asp-217, from about Pro-239 to about Leu-264, and from about Ala-283 to about Pro-298 (SEQ ID NO:2). Antigenic regions of TNFR-6 $\beta$ , include from about Ala-31 to about Thr-46, from about Phe-57 to about Gln-80, from about Glu-86 to about His-106, from about Thr-108 to about Phe-119, from about His-129 to about Val-138, and from about Gly-142 to about Pro-166 (SEQ ID NO:4). These polypeptide fragments have been

determined to bear antigenic epitopes of the TNFR-6 alpha and TNFR-6 beta polypeptides by the analysis of the Jameson-Wolf antigenic index.

The data presented in Figures 4 and 5 are also represented in tabular form in Tables I and II, respectively. The columns are labeled with the headings "Res", "Position", and Roman Numerals I-XIV. The column headings refer to the following features of the amino acid sequence presented in Figure 4, (Table I) and Figure 5 (Table II): "Res": amino acid residue of SEQ ID NO:2 (Figure 1) or SEQ ID NO:4 (Figure 2); "Position": position of the corresponding residue within of SEQ ID NO:2 (Figure 1) or SEQ ID NO:4 (Figure 2); I: Alpha, Regions - Garnier-Robson; II: Alpha, Regions - Chou-Fasman; III: Beta, Regions - Garnier-Robson; IV: Beta, Regions - Chou-Fasman; V: Turn, Regions - Garnier-Robson; VI: Turn, Regions - Chou-Fasman; VII: Coil, Regions - Garnier-Robson; VIII: Hydrophilicity Plot - Kyte-Doolittle; IX: Hydrophobicity Plot - Hopp-Woods; X: Alpha, Amphipathic Regions - Eisenberg; XI: Beta, Amphipathic Regions - Eisenberg; XII: Flexible Regions - Karplus-Schulz; XIII: Antigenic Index - Jameson-Wolf; and XIV: Surface Probability Plot - Emini.

Figure 6 shows the nucleotide sequences of HELDI06R (SEQ ID NO:17) and HCEOW38R (SEQ ID NO:18) which are related to SEQ ID NOS:1 and 3.

Figures 7A-B show TNFR6 alpha blocking of Fas ligand mediated cell death. Jurkat T-cells were treated with a combination of Fas ligand and TNFR 6 alpha Fc receptor for 16 hours. To measure the levels of viable cells after treatment, cells were incubated for 5 hours with 10% ALOMAR blue and examined spectrophotometrically at OD 570nm-630nm. All samples were tested in triplicate. TNFR6 alpha-Fc appears to block Fas ligand mediated apoptosis of Jurkat cells in a dose dependent manner as effectively as Fas ligand.

### **Detailed Description**

The present invention provides isolated nucleic acid molecules comprising, or alternatively consisting of, a polynucleotide encoding a TNFR-6 $\alpha$  or -6 $\beta$  polypeptide, generically "TNFR polypeptide(s)" having the amino acid sequence shown in SEQ ID NOS:2 and 4, respectively, which were determined by sequencing cloned cDNAs. The nucleotide sequences shown in Figures 1 and 2 (SEQ ID NOS:1 and 3) were obtained by sequencing the HPHAE52 and HTPCH84 clones, respectively, which were deposited on November 22, 1996 at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 and given accession numbers ATCC 97810 and 97809, respectively. The deposited clones are contained in the pBluescript SK(-) plasmid (Stratagene, La Jolla, CA).

The TNFR-6 alpha and TNFR-6 beta proteins of the present invention are splice variants which share an identical nucleotide and amino acid sequence over the N-terminal 142 residues of the respective proteins. The amino acid sequences of these proteins are about 23% similar to and share multiple conserved cysteine rich domains with the translation product of the human TNFR-2 mRNA (Figure 3) (SEQ ID NO:6). Importantly, these proteins share substantial sequence similarity over a polypeptide sequence including four repeated cysteine rich motifs with significant intersubunit homology. TNFR-2 is thought to exclusively mediate human T-cell proliferation by TNF (PCT WO/94/09137).

### **Nucleic Acid Molecules**

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc., Foster City, CA), and all amino acid sequences of polypeptides encoded by DNA

molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

By "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U), where each thymidine deoxyribonucleotide (T) in the specified deoxyribonucleotide sequence is replaced by the ribonucleotide uridine (U).

Using the information provided herein, such as the nucleotide sequences in Figures 1 and <sup>2A-B</sup> (SEQ ID NOS:1 and 3), a nucleic acid molecule of the present invention encoding a TNFR polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the TNFR-6 $\alpha$  and TNFR-6 $\beta$  clones (Figures 1 and <sup>2A-B</sup>, respectively) were identified in cDNA

libraries from the following tissues: endothelial cells, keratinocytes, normal prostate tissue, and prostate tumor tissue.

The determined nucleotide sequences of the TNFR cDNAs of Figures 1 and <sup>2A-3</sup>~~A~~ (SEQ ID NOS:1 and 3) contain open reading frames encoding proteins of 300 and 170 amino acid residues, with an initiation codon at nucleotide positions 25-27 and 73-75 of the nucleotide sequences in Figures 1 and <sup>2A-3</sup>~~A~~ (SEQ ID NOS:1 and 3), respectively.

The open reading frames of the TNFR-6 $\alpha$  and TNFR-6 $\beta$  genes share sequence homology with the translation product of the human mRNA for TNFR-2, including the soluble extracellular domain of about residues 31-283 of SEQ ID NO:2 and 31-166 of SEQ ID NO:4, respectively.

As one of ordinary skill would appreciate, due to the possibilities of sequencing errors discussed above, the actual complete TNFR polypeptides encoded by the deposited cDNAs, which comprise about 300 and 170 amino acids, may be somewhat longer or shorter. More generally, the actual open reading frames may be anywhere in the range of  $\pm 20$  amino acids, more likely in the range of  $\pm 10$  amino acids, of that predicted from the first methionine codon from the N-terminus shown in Figures 1 and <sup>2A-3</sup>~~A~~ (SEQ ID NOS:1 and 3), which is in-frame with the translated sequences shown in each respective figure. It will further be appreciated that, depending on the analytical criteria used for identifying various functional domains, the exact "address" of the extracellular and transmembrane domain(s) of the TNFR polypeptides may differ slightly from the predicted positions above. For example, the exact location of the extracellular domain or antigenic regions in SEQ ID NO:2 and SEQ ID NO:4 may vary slightly (e.g., the address may "shift" by about 1 to about 20 residues, more likely about 1 to about 5 residues) depending on the criteria used to define the domains and antigenic regions. In any event, as discussed further below, the invention further provides polypeptides having

various residues deleted from the N-terminus of the complete polypeptide, including polypeptides lacking one or more amino acids from the N-terminus of the extracellular domain described herein, which constitute soluble forms of the extracellular domains of the TNFR-6 $\alpha$  and TNFR-6 $\beta$  proteins.

5           The amino acid sequences of the complete TNFR proteins include a leader sequence and a mature protein, as shown in SEQ ID NOS:2 and 4. More in particular, the present invention provides nucleic acid molecules encoding mature forms of the TNFR proteins. Thus, according to the signal hypothesis, once export of the growing protein chain across the rough endoplasmic reticulum has been initiated, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the complete polypeptide to produce a secreted "mature" form of the protein. Most mammalian cells and even insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species of the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding a mature TNFR polypeptide having the amino acid sequence encoded by a cDNA clone identified as ATCC Deposit No. 97810 or 97809. By the "mature TNFR polypeptides having the amino acid sequence encoded by a cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810, or 97809" is meant the mature form(s) of the protein produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the human DNA sequence of the clone contained in the deposited vector.

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In addition, methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For

instance, the method of McGeoch (*Virus Res.* 3:271-286 (1985)) uses the information from a short N-terminal charged region and a subsequent uncharged region of the complete (uncleaved) protein. The method of von Heinje (*Nucleic Acids Res.* 14:4683-4690 (1986)) uses the information from the residues surrounding the cleavage site, typically residues -13 to +2 where +1 indicates the amino terminus of the mature protein. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 75-80% (von Heinje, *supra*). However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the deduced amino acid sequence of the complete TNFR polypeptides were analyzed by a computer program "PSORT", available from Dr. Kenta Nakai of the Institute for Chemical Research, Kyoto University (see K. Nakai and M. Kanehisa, *Genomics* 14:897-911 (1992)), which is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis of the TNFR amino acid sequences by this program provided the following results: TNFR-6 $\alpha$  & TNFR-6 $\beta$  encode mature polypeptides having the amino acid sequences of residues 31-300 and 31-170 of SEQ ID NOS:2 and 4, respectively.

In certain preferred embodiments, TNFR-6 $\alpha$  & TNFR-6 $\beta$  encode mature polypeptides having the amino acid sequences of residues 31-299 and 31-169 of SEQ ID NOS:2 and 4, respectively.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded.

Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. However, a nucleic acid contained in a clone that is a member of a mixed clone library (e.g., a genomic or cDNA library) and that has not been isolated from other clones of the library (e.g., in the form of a homogeneous solution containing the clone without other members of the library) or a chromosome isolated or removed from a cell or a cell lysate (e.g., a "chromosome spread", as in a karyotype), is not "isolated" for the purposes of this invention. As discussed further herein, isolated nucleic acid molecules according to the present invention may be produced naturally, recombinantly, or synthetically.

Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) with an initiation codon at positions 25-27 and 73-75 of the nucleotide sequences shown in SEQ ID NOS:1 and 3, respectively.

Also included are DNA molecules comprising the coding sequence for the predicted mature TNFR polypeptides shown at positions 31-300 and 31-170 of SEQ ID NOS:2 and 4, respectively.

Also included are DNA molecules comprising the coding sequence for the predicted mature TNFR polypeptides shown at positions 31-299 and 31-169 of SEQ ID NOS:2 and 4, respectively.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode a TNFR protein. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

In another aspect, the invention provides isolated nucleic acid molecules encoding a TNFR polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809. Preferably, this nucleic acid molecule will encode the mature polypeptide encoded by the above-described deposited cDNA clone.

The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in Figure 1 or <sup>Figures 2A-3</sup> (SEQ ID NO:1 or 3) or the nucleotide sequence of the TNFR cDNAs contained in the above-described deposited clones, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful, for example, as probes for gene mapping by *in situ* hybridization with chromosomes, and for detecting expression of the TNFR genes in human tissue, for instance, by Northern blot analysis.

The present invention is further directed to nucleic acid molecules encoding portions of the nucleotide sequences described herein as well as to fragments of the isolated nucleic acid molecules described herein. In particular, the invention provides polynucleotides having a nucleotide sequence representing the portion of SEQ ID NO:1 or 3 which consist of positions 25-924 and 73-582 of SEQ ID NOS:1 and 3, respectively. Also contemplated are

polynucleotides encoding TNFR polypeptides which lack an amino terminal methionine such polynucleotides having a nucleotide sequence representing the portion of SEQ ID NOS:1 and 3 which consist of positions 28-924 and 76-582, respectively. Polypeptides encoded by such polynucleotides are also provided, such polypeptides comprising an amino acid sequence at positions 5 2-300 and 2-170 of SEQ ID NOS:2 and 4, respectively.

In addition, the invention provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NOS:1 and 3 as follows: HELDI06R (SEQ ID NO:17) and HCEOW38R (SEQ ID NO:18) are related to both SEQ ID NOS:1 and 3. Preferred are polynucleotide fragments of SEQ ID NOS:1 and 3 which are not SEQ ID NO:17 or 18 or subfragments of either SEQ ID NO:17 or 18. The sequences of HELDI06R and HCEOW38R are shown in Figure 6.

More generally, by a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNA or the nucleotide sequence shown in Figures 1 or <sup>Figures 2A-B</sup> (SEQ ID NOS:1 or 3) is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length. These fragments have numerous uses, which include, but are not limited to, as diagnostic probes and primers as discussed herein. Of course, larger fragments 50-300 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNAs or as shown in Figures 1 and <sup>2A-B</sup> (SEQ ID NOS:1 and 3). Especially preferred are fragments comprising at least 500 nucleotides which are at least 80%, 85%, 90%, 92%, or 95% identical to 500 contiguous nucleotides shown in SEQ ID NO:1. By a fragment at least about 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of a deposited cDNA or the nucleotide sequence as shown in Figures 1 and <sup>2A-B</sup> (SEQ ID NOS:1 and 3). In

this context "about" includes the particularly recited size, and those sizes that are larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini. Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of the TNFR polypeptides as identified in Figures 4 and 5 and described in more detail below.

Representative examples of TNFR-6 $\alpha$  nucleic acid fragments of the invention include, for example, fragments that comprise, or alternatively, consist of, a sequence from about nucleotide 1 to about nucleotide 25, about nucleotide 26 to about nucleotide 75, about nucleotide 76 to about nucleotide 114, about nucleotide 115 to about nucleotide 162, about nucleotide 163 to about nucleotide 216, about nucleotide 217 to about nucleotide 267, about nucleotide 268 to about nucleotide 318, about nucleotide 319 to about nucleotide 369, about nucleotide 370 to about nucleotide 420, about nucleotide 421 to about nucleotide 471, about nucleotide 472 to about nucleotide 522, about nucleotide 523 to about nucleotide 573, about nucleotide 574 to about nucleotide 625, about nucleotide 626 to about nucleotide 675, about nucleotide 676 to about nucleotide 714, about nucleotide 715 to about nucleotide 765, about nucleotide 766 to about nucleotide 816, about nucleotide 817 to about nucleotide 867, about nucleotide 868 to about nucleotide 924, about nucleotide 925 to about nucleotide 975 of SEQ ID NO:1, or the complementary strand thereto, or the cDNA contained in the plasmid deposited as ATCC Deposit No. 97810. In this context "about" includes the particularly recited ranges, and those ranges that are larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini.

In specific embodiments, the nucleic acid fragments of the invention comprise, or alternatively, consist of, a polynucleotide sequence encoding amino acid residues 100 to 150, 150 to 200, 200 to 300, 220 to 300, 240 to 300, 250 to 300, 260 to 300, and/or 280 to 300, of SEQ ID NO:2, or the

complementary strand thereto. Polynucleotides that hybridize to these polynucleotide fragments are also encompassed by the invention.

Representative examples of TNFR--6 $\beta$  nucleic acid fragments of the invention include, for example, fragments that comprise, or alternatively, consist of, a sequence from about nucleotide 1 to about nucleotide 36, about nucleotide 37 to about nucleotide 72, about nucleotide 73 to about nucleotide 123, about nucleotide 124 to about nucleotide 175, about nucleotide 176 to about nucleotide 216, about nucleotide 217 to about nucleotide 267, about nucleotide 268 to about nucleotide 318, about nucleotide 319 to about nucleotide 369, about nucleotide 370 to about nucleotide 420, about nucleotide 421 to about nucleotide 471, about nucleotide 472 to about nucleotide 522, about nucleotide 523 to about nucleotide 582, about nucleotide 583 to about nucleotide 622, about nucleotide 623 to about nucleotide 682, about nucleotide 683 to about nucleotide 750, about nucleotide 751 to about nucleotide 800, about nucleotide 801 to about nucleotide 850, about nucleotide 851 to about nucleotide 900, about nucleotide 901 to about nucleotide 950, about nucleotide 951 to about nucleotide 1000, about nucleotide 1001 to about nucleotide 1050, about nucleotide 1051 to about nucleotide 1100, about nucleotide 1101 to about nucleotide 1150, about nucleotide 1151 to about nucleotide 1200, about nucleotide 1201 to about nucleotide 1250, about nucleotide 1251 to about nucleotide 13000, about nucleotide 1301 to about nucleotide 1350, about nucleotide 1351 to about nucleotide 1400, about nucleotide 1401 to about nucleotide 1450, about nucleotide 1451 to about nucleotide 1500, about nucleotide 1501 to about nucleotide 1550, about nucleotide 1551 to about nucleotide 1600 about nucleotide 1601 to about nucleotide 1650, about nucleotide 1651 to about nucleotide 1667 of SEQ ID NO:3, or the complementary strand thereto, or the cDNA contained in the plasmid deposited as ATCC Deposit No. 97809. In this context "about" includes the particularly recited ranges, and those ranges that are larger or smaller by several

(5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini.

In specific embodiments, the nucleic acid fragments of the invention comprise, or alternatively, consist of, a polynucleotide sequence encoding amino acid residues 50 to 100, 100 to 170, 110 to 170, 130 to 170, 140 to 170, 150 to 170, and/or 160 to 170, of SEQ ID NO:4, or the complementary strand thereto. Polynucleotides that hybridize to these polynucleotide fragments are also encompassed by the invention.

Preferably, the polynucleotide fragments of the invention encode a polypeptide which demonstrates a TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  functional activity. By a polypeptide demonstrating "functional activity" is meant, a polypeptide capable of displaying one or more known functional activities associated with a complete (full-length) or mature TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptide. Such functional activities include, but are not limited to, biological activity (e.g., inhibition or reduction of FasL mediated apoptosis, inhibition or reduction of AIM-II mediated apoptosis), antigenicity [ability to bind (or compete with a TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptide for binding) to an anti-TNFR-6 $\alpha$  antibody and/or anti-TNFR -6 $\beta$  antibody], immunogenicity (ability to generate antibody which binds to a TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptide), ability to form multimers with TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptides of the invention, and ability to bind to a receptor or ligand for a TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptide (e.g., Fas ligand and/or AIM-II (International application publication number WO 97/34911, published September 25, 1997)).

The functional activity of TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptides, and fragments, variants derivatives, and analogs thereof, can be assayed by various methods.

For example, in one embodiment where one is assaying for the ability to bind or compete with complete (full-length) or mature TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  polypeptide for binding to anti-TNFR-6 $\alpha$  and/or anti-TNFR-6 $\beta$  antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In another embodiment, where a TNF-ligand is identified (e.g., Fas Ligand and/or AIM-II (International application publication number WO 97/34911, published September 25, 1997)), or the ability of a polypeptide fragment, variant or derivative of the invention to multimerize is being evaluated, binding can be assayed, e.g., by means well-known in the art, such as, for example, reducing and non-reducing gel chromatography, protein affinity chromatography, and affinity blotting. See generally, Phizicky, E., *et al.*, *Microbiol. Rev.* 59:94-123 (1995). In another embodiment, physiological correlates of TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  binding to its substrates (signal transduction) can be assayed.

In addition, assays described herein (e.g., see Examples 7 -9) and otherwise known in the art may routinely be applied or modified to measure the ability of TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptides and fragments, variants derivatives and analogs thereof, to elicit TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  related biological activity (e.g., to inhibit or reduce FasL mediated apoptosis *in vitro or in vivo*, or to inhibit or reduce AIM-II mediated apoptosis *in vitro or in vivo*).

For example, the ability of TNFR polypeptides of the invention to reduce or block FasL mediated apoptosis can be assayed using a Fas expressing T-cell line, such as Jurkat. In this assay, Jurkat cells treated with soluble FasL undergo apoptosis. Pretreatment of cells with TNFR and/or TNFR agonists prior to addition of FasL protects cells from undergoing apoptosis and results in a reduced level of apoptosis when compared to that observed when the same concentration of soluble FasL is contacted with the same concentration of the Fas expressing cells in the absence of the TNFR polypeptide or TNFR agonist. Alternatively mixing of the FasL protein with TNFR and/or TNFR agonist will also block the ability of FasL to bind the Jurkat cells and mediate apoptosis (see, e.g., Example 9).

In contrast, TNFR antagonists of the invention block TNFR mediated inhibition of FasL mediated apoptosis. Accordingly, TNFR antagonists of the invention can be assayed, for example, by combining the mature TNFR (known to bind FasL), the TNFR antagonist to be tested, and soluble FasL, and contacting this combination with the Fas expressing cell line. TNFR antagonists reduce or block TNFR mediated inhibition of FasL mediated apoptosis. Accordingly, Fas expressing T cells contacted with mature TNFR, TNFR antagonist and soluble FasL exhibit elevated apoptosis levels when compared with the same concentration of Fas expressing cells that have been

contacted with the same concentrations of mature TNFR and FasL in the absence of the TNFR antagonist.

Apoptosis can be measured, for example, by increased staining with Annexin, which selectively binds apoptotic cells. In another example, the decrease in cell numbers due to apoptosis can be detected by a decrease in ALOMAR blue staining which detects viable cells.

Other methods will be known to the skilled artisan and are within the scope of the invention.

In additional embodiments, the polynucleotides of the invention encode functional attributes of TNFR-6 $\alpha$  and/or TNFR -6 $\beta$ . Preferred embodiments of the invention in this regard include fragments that comprise alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet forming regions ("beta-regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions and high antigenic index regions of TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  polypeptides.

Certain preferred regions in this regard are set out in Figure 4 (Table I) and Figure 5 (Table II). The data presented in Figures 4 and Figure 5 and that presented in Table I and Table II, respectively, merely present a different format of the same results obtained when the amino acid sequence of SEQ ID NO:2 and the amino acid sequence of SEQ ID NO:4 is analyzed using the default parameters of the DNA\*STAR computer algorithm.

The above-mentioned preferred regions set out in Figure 4 (Table I) and Figure 5 (Table II) include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence set out in Figure 1 and <sup>Figures 2A-B</sup> ~~Figure 2~~. As set out in Figure 4 (Table I) and Figure 5 (Table II), such preferred regions include Garnier-Robson alpha-regions, beta-regions,

turn-regions, and coil-regions, Chou-Fasman alpha-regions, beta-regions, and coil-regions, Kyte-Doolittle hydrophilic regions, Eisenberg alpha- and beta-amphipathic regions, Karplus-Schulz flexible regions, Emini surface-forming regions and Jameson-Wolf regions of high antigenic index.

5 Among highly preferred polynucleotides in this regard are those that encode polypeptides comprising regions of TNFR-6<sub>α</sub> and/or TNFR-6<sub>β</sub> that combine several structural features, such as several (e.g., 1, 2, 3 , or 4) of the features set out above.

10 Additionally, the data presented in columns VIII, IX, XIII, and XIV of Tables I and II can routinely be used to determine regions of TNFR-6<sub>α</sub> which exhibit a high degree of potential for antigenicity. Regions of high antigenicity are determined from the data presented in columns VIII, IX, XIII, and/or XIV by choosing values which represent regions of the polypeptide which are likely to be exposed on the surface of the polypeptide in an environment in which antigen 15 recognition may occur in the process of initiation of an immune response.

**Table I**

	Res Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Met 1	.	.	B	.	.	.	.	0.06	0.09	*	.	.	-0.10	0.60
	Arg 2	.	.	B	.	.	.	.	0.10	-0.34	*	.	.	0.50	0.82
	Ala 3	.	.	B	.	.	.	.	0.28	-0.34	*	.	.	0.50	0.63
	Leu 4	A	.	.	.	.	.	.	0.32	-0.34	*	.	.	0.50	0.99
10	Glu 5	A	.	.	.	.	.	.	-0.10	-0.53	*	.	F	0.95	0.50
	Gly 6	.	.	.	.	.	T	C	0.20	0.16	*	.	F	0.45	0.41
	Pro 7	.	.	.	.	T	T	.	-0.72	0.04	*	.	F	0.65	0.66
	Gly 8	.	.	.	.	T	T	.	-0.94	0.04	.	.	F	0.65	0.32
	Leu 9	A	.	.	.	.	T	.	-0.80	0.73	.	.	.	-0.20	0.26
15	Ser 10	A	A	.	.	.	.	.	-1.61	0.87	.	.	.	-0.60	0.09
	Leu 11	.	A	B	.	.	.	.	-2.12	1.13	.	.	.	-0.60	0.08
	Leu 12	.	A	B	.	.	.	.	-2.72	1.34	.	.	.	-0.60	0.07
	Cys 13	.	A	B	.	.	.	.	-2.97	1.34	.	.	.	-0.60	0.04
	Leu 14	.	A	B	.	.	.	.	-2.97	1.46	.	.	.	-0.60	0.05
20	Val 15	.	A	B	.	.	.	.	-2.88	1.46	.	.	.	-0.60	0.05
	Leu 16	.	A	B	.	.	.	.	-2.66	1.20	.	.	.	-0.60	0.15
	Ala 17	.	A	B	.	.	.	.	-2.66	1.13	.	.	.	-0.60	0.18
	Leu 18	.	A	B	.	.	.	.	-2.80	1.13	.	.	.	-0.60	0.20
	Pro 19	A	A	.	.	.	.	.	-2.20	1.17	.	.	.	-0.60	0.20
25	Ala 20	.	A	B	.	.	.	.	-2.20	0.91	.	*	.	-0.60	0.31
	Leu 21	.	A	B	.	.	.	.	-1.60	1.06	.	*	.	-0.60	0.28
	Leu 22	.	A	B	.	.	.	.	-1.60	0.80	.	.	.	-0.60	0.28
	Pro 23	.	A	B	.	.	.	.	-1.64	0.87	.	*	.	-0.60	0.28
	Val 24	.	.	B	.	.	.	.	-1.32	1.01	.	*	.	-0.40	0.25
30	Pro 25	.	.	B	.	.	.	.	-1.08	0.33	*	.	.	-0.10	0.60
	Ala 26	.	.	B	B	.	.	.	-1.12	0.07	.	.	.	-0.30	0.38
	Val 27	.	.	B	B	.	.	.	-0.90	0.29	*	*	.	-0.30	0.38
	Arg 28	.	.	B	B	.	.	.	-0.69	0.14	*	*	.	-0.30	0.25
	Gly 29	.	.	B	B	.	.	.	-0.14	-0.29	*	*	.	0.30	0.43
35	Val 30	.	.	B	B	.	.	.	-0.14	-0.30	*	*	.	0.30	0.83
	Ala 31	.	.	B	B	.	.	.	0.13	-0.51	*	*	.	0.60	0.66
	Glu 32	.	.	B	.	.	.	.	0.74	-0.03	*	*	F	0.65	0.96
	Thr 33	.	.	B	.	.	T	.	0.42	0.30	*	*	F	0.40	2.02
	Pro 34	.	.	B	.	.	T	T	0.48	0.09	*	*	F	0.80	3.10
40	Thr 35	.	.	.	.	T	T	.	1.44	0.50	*	.	F	0.50	1.88
	Tyr 36	.	.	.	.	.	T	C	2.03	0.50	*	.	.	0.15	2.55
	Pro 37	.	.	.	.	T	.	.	1.44	0.01	*	.	.	0.45	2.76
	Trp 38	.	A	.	.	.	.	C	1.76	0.09	*	.	.	0.05	1.93
	Arg 39	.	A	B	.	.	.	.	1.66	-0.40	*	.	F	0.60	2.13
45	Asp 40	A	A	.	.	.	.	.	1.62	-0.67	*	.	F	0.90	1.99
	Ala 41	.	A	A	.	.	.	C	1.87	-0.67	*	*	F	1.10	1.87
	Glu 42	A	A	.	.	.	.	.	2.19	-1.59	*	*	F	0.90	1.66
	Thr 43	A	A	A	.	.	.	.	1.67	-1.59	*	.	F	0.90	1.94
	Gly 44	.	A	A	.	T	.	.	0.70	-0.90	*	.	F	1.30	1.59
50	Glu 45	A	A	.	.	.	.	.	0.03	-0.76	*	*	F	0.75	0.68
	Arg 46	A	A	A	.	.	.	.	0.03	-0.19	*	.	F	0.45	0.25
	Leu 47	A	A	A	.	.	.	.	0.03	-0.17	*	.	.	0.30	0.26
	Val 48	.	A	B	.	.	.	.	-0.32	-0.20	.	.	.	0.30	0.26
	Cys 49	.	A	B	.	.	.	.	-0.19	0.37	.	.	.	-0.30	0.07
55	Ala 50	.	A	B	.	.	.	.	-0.40	0.80	.	.	.	-0.60	0.13
	Gln 51	.	A	B	.	.	.	.	-0.86	0.54	.	.	.	-0.60	0.28
	Cys 52	.	A	B	.	.	.	.	-0.36	0.33	.	.	.	-0.30	0.51
	Pro 53	.	.	.	.	.	T	C	-0.20	0.24	.	.	F	0.45	0.73
	Pro 54	.	.	.	.	T	T	.	-0.39	0.53	.	.	F	0.35	0.36
	Gly 55	.	.	.	.	T	T	.	0.20	0.77	*	.	F	0.35	0.50
60	Thr 56	.	.	B	.	.	T	.	0.31	0.60	.	.	F	-0.05	0.56

**Table I**

	Res Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Phe 57	.	.	B	B	.	.	.	0.77	0.17	*	.	F	-0.15	0.71
	Val 58	.	.	B	B	.	.	.	0.31	0.17	*	.	.	0.19	1.12
	Gln 59	.	.	B	B	.	.	.	0.63	0.31	*	.	F	0.53	0.41
10	Arg 60	.	.	B	.	.	T	.	1.09	-0.17	*	.	F	1.87	0.94
	Pro 61	.	.	B	.	.	T	.	1.40	-0.96	*	.	F	2.66	2.47
	Cys 62	.	.	.	.	T	T	.	1.80	-1.60	*	.	F	3.40	2.38
	Arg 63	.	.	.	.	T	T	.	2.44	-1.61	*	.	F	3.06	1.63
	Arg 64	.	.	.	.	T	.	.	2.13	-1.19	*	.	F	2.77	1.63
15	Asp 65	.	.	.	.	T	.	.	1.71	-1.13	*	.	F	2.68	4.39
	Ser 66	.	.	.	.	T	T	.	1.26	-1.21	.	.	F	2.79	3.24
	Pro 67	.	.	.	.	T	T	.	1.58	-0.64	.	.	F	2.55	0.89
	Thr 68	.	.	.	.	T	T	.	1.26	-0.21	.	.	F	2.50	0.52
	Thr 69	.	.	.	.	T	T	.	0.48	0.21	.	.	F	1.65	0.61
20	Cys 70	.	.	.	.	T	.	.	0.27	0.40	.	.	F	1.14	0.21
	Gly 71	.	.	.	.	T	T	.	0.36	0.40	*	*	F	1.33	0.22
	Pro 72	.	.	.	.	T	T	.	0.68	0.34	*	.	F	1.62	0.24
	Cys 73	.	.	.	.	.	T	C	0.96	-0.14	*	.	F	2.01	0.88
	Pro 74	.	.	.	.	.	T	C	1.02	-0.21	*	.	F	2.40	1.21
25	Pro 75	.	.	.	.	T	T	.	1.38	0.11	*	*	F	1.76	1.23
	Arg 76	.	.	.	.	T	T	.	1.72	0.17	*	*	F	1.52	3.30
	His 77	.	.	B	.	.	T	.	1.23	0.00	*	*	F	0.88	3.70
	Tyr 78	.	.	B	.	.	T	.	1.61	0.36	*	*	.	0.49	2.07
	Thr 79	.	.	B	.	.	.	.	1.82	0.84	*	.	.	-0.25	1.11
30	Gln 80	.	.	B	.	.	.	.	1.79	1.24	*	*	.	-0.25	1.31
	Phe 81	.	.	.	.	T	.	.	0.87	1.50	*	*	.	0.15	1.31
	Trp 82	.	.	.	.	T	.	.	0.90	1.43	*	.	.	0.00	0.75
	Asn 83	.	A	.	.	T	.	.	1.26	0.94	*	.	.	-0.20	0.75
	Tyr 84	.	A	.	.	T	.	.	0.90	0.54	*	*	.	-0.05	1.70
35	Leu 85	.	A	.	.	T	.	.	1.01	0.33	*	*	.	0.38	0.87
	Glu 86	.	A	.	.	T	.	.	1.47	-0.59	*	*	.	1.71	1.05
	Arg 87	.	A	.	.	T	.	.	1.09	-0.23	.	*	.	1.69	1.05
	Cys 88	.	.	.	.	T	T	.	1.09	-0.41	.	*	.	2.22	0.69
	Arg 89	.	.	.	.	T	T	.	0.48	-0.70	.	*	.	2.80	0.64
40	Tyr 90	.	.	.	.	T	T	.	0.48	-0.06	.	*	.	2.22	0.24
	Cys 91	.	.	.	.	T	T	.	-0.19	0.63	.	*	.	1.04	0.37
	Asn 92	.	.	B	B	.	.	.	-0.64	0.63	.	*	.	-0.04	0.10
	Val 93	.	.	B	B	.	.	.	0.02	1.06	.	*	.	-0.32	0.06
	Leu 94	.	.	B	B	.	.	.	0.02	0.30	.	.	.	-0.30	0.21
45	Cys 95	.	.	B	.	.	T	.	0.27	-0.27	.	.	.	0.70	0.25
	Gly 96	.	.	.	.	T	.	C	0.93	-0.67	.	.	F	1.35	0.59
	Glu 97	A	.	.	.	.	T	.	0.93	-1.31	.	.	F	1.30	1.24
	Arg 98	A	.	.	.	.	T	.	1.20	-2.00	.	*	F	1.30	4.00
	Glu 99	A	A	.	.	.	.	.	2.12	-2.07	.	*	F	0.90	4.08
50	Glu 100	A	A	.	.	.	.	.	2.20	-2.50	.	*	F	0.90	4.61
	Glu 101	A	A	.	.	.	.	.	1.88	-2.00	.	*	F	0.90	2.38
	Ala 102	A	A	.	.	.	.	.	1.84	-1.43	.	.	F	0.75	0.74
	Arg 103	A	A	.	.	.	.	.	1.14	-0.93	.	.	.	0.60	0.58
	Ala 104	A	A	.	.	.	.	.	0.83	-0.43	.	*	.	0.30	0.34
	Cys 105	A	A	.	.	.	.	.	0.80	0.06	.	*	.	-0.30	0.48
55	His 106	A	A	.	.	.	.	.	0.80	0.06	*	*	.	-0.30	0.34
	Ala 107	A	A	.	.	.	.	.	1.50	0.46	*	*	.	-0.60	0.53
	Thr 108	A	A	.	.	.	.	.	0.80	-0.04	*	*	.	0.45	1.95
	His 109	.	A	.	.	.	T	.	0.72	-0.11	*	.	.	1.13	1.45
60	Asn 110	.	A	.	.	T	.	.	1.50	-0.04	*	.	.	1.26	0.77
	Arg 111	.	A	.	.	T	.	.	0.87	-0.54	*	*	.	1.99	1.04
	Ala 112	.	A	.	.	T	.	.	1.57	-0.46	*	.	.	1.82	0.41

**Table I (continued)**

	Res Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Cys 113	.	.	.		T	T	.	1.57	-0.96	.	*	.	2.80	0.50
	Arg 114	.	.	B	.	T	T	.	1.26	-0.87	*	*	.	2.12	0.37
	Cys 115	.	.	.		T	T	.	0.56	-0.44	*	*	.	1.94	0.36
10	Arg 116	.	.	.		T	T	.	-0.26	-0.16	.	*	.	1.66	0.58
	Thr 117	.	A	.	B	T	.	.	-0.26	0.06	.	*	F	0.53	0.26
	Gly 118	.	A	B	B	T	.	.	0.38	0.56	.	*	.	-0.20	0.49
	Phe 119	.	A	B	B	.	.	.	-0.32	0.49	.	*	.	-0.60	0.34
	Phe 120	.	A	B	B	.	.	.	-0.00	0.99	.	*	.	-0.60	0.24
15	Ala 121	A	A	.	B	.	.	.	-0.81	0.93	.	*	.	-0.60	0.24
	His 122	A	A	.	.	.	.	.	-1.17	1.29	.	.	.	-0.60	0.24
	Ala 123	A	A	.	.	.	.	.	-1.63	1.07	.	*	.	-0.60	0.15
	Gly 124	A	A	.	.	.	.	.	-0.93	0.97	.	*	.	-0.60	0.12
	Phe 125	A	A	.	.	.	.	.	-0.27	0.47	.	*	.	-0.60	0.15
20	Cys 126	A	A	.	.	.	.	.	-0.27	0.47	.	*	.	-0.60	0.20
	Leu 127	A	A	.	.	.	.	.	-0.53	0.47	.	.	.	-0.60	0.21
	Glu 128	A	A	.	.	.	.	.	-0.61	0.43	.	.	.	-0.60	0.32
	His 129	.	.	.		T	T	.	-0.48	0.21	.	.	.	0.50	0.32
	Ala 130	.	.	.		T	T	.	0.01	0.07	.	.	.	0.63	0.61
25	Ser 131	.	.	.		T	T	C	0.33	-0.19	.	.	.	1.36	0.54
	Cys 132	.	.	.	.	T	C	0.56	0.24	.	.	.	0.69	0.39	
	Pro 133	.	.	.	.	T	C	0.21	0.24	.	.	F	0.97	0.39	
	Pro 134	.	.	.		T	T	.	-0.61	0.17	.	F	.	1.30	0.29
	Gly 135	.	.			T	T	.	-0.91	0.43	.	F	.	0.87	0.40
30	Ala 136	.	B	.		T	.	.	-1.20	0.54	.	.	.	0.19	0.18
	Gly 137	.	B	B	.	.	.	.	-0.74	0.61	.	.	.	-0.34	0.12
	Val 138	.	B	B	.	.	.	.	-0.88	0.61	.	.	.	-0.47	0.19
	Ile 139	.	B	B	.	.	.	.	-0.98	0.61	.	.	.	-0.60	0.18
	Ala 140	.	B	B	.	.	.	.	-0.84	0.60	.	.	.	-0.60	0.27
35	Pro 141	.	B	.		T	.	.	-0.56	0.60	.	F	.	-0.25	0.55
	Gly 142	.	.	.		T	.	C	-0.21	0.34	.	F	.	0.88	1.06
	Thr 143	.	.	.		T	C	0.64	0.06	.	F	.	1.16	1.82	
	Pro 144	.	.	.		T	C	1.22	-0.04	.	F	.	2.04	1.89	
	Ser 145	.	.			T	T	.	1.81	0.01	.	F	.	1.92	2.76
40	Gln 146	.	.			T	T	.	1.36	-0.01	.	F	.	2.80	3.31
	Asn 147	.	.			T	T	.	1.70	0.07	.	F	.	1.92	1.15
	Thr 148	.	.			T	T	.	1.80	0.04	.	F	.	1.64	1.48
	Gln 149	.	.			T	T	.	1.34	0.09	.	F	.	1.36	1.32
	Cys 150	.	B	.		T	.	.	1.43	0.26	.	F	.	0.53	0.44
45	Gln 151	.	B	.		.	.	.	1.22	0.29	.	F	.	0.05	0.47
	Pro 152	.	B	.		.	.	.	0.88	0.23	*	F	.	0.05	0.42
	Cys 153	.	B	.		.	.	.	0.88	0.26	*	F	.	0.05	0.78
	Pro 154	.	B	.		T	.	.	0.18	0.17	*	F	.	0.25	0.65
	Pro 155	.	.			T	T	.	0.54	0.56	*	F	.	0.35	0.36
50	Gly 156	.	.			T	T	.	-0.04	0.51	*	F	.	0.35	0.91
	Thr 157	.	B	.		T	.	.	-0.13	0.44	.	F	.	-0.05	0.59
	Phe 158	.	B	.		.	.	.	0.23	0.40	.	F	.	-0.25	0.51
	Ser 159	.	B	.		.	.	.	0.14	0.36	.	F	.	0.39	0.70
	Ala 160	.	B	.		.	.	.	0.06	0.31	.	F	.	0.73	0.65
55	Ser 161	.	.	.		T	C	0.10	0.21	.	F	.	1.62	1.00	
	Ser 162	.	.	.		T	C	0.41	-0.19	.	F	.	2.56	1.00	
	Ser 163	.	.	.		T	T	.	1.11	-0.57	.	F	.	3.40	1.72
	Ser 164	.	.	.		T	T	.	0.74	-0.67	.	F	.	3.06	2.22
	Ser 165	.	.	.		T	.	.	1.33	-0.49	.	F	.	2.07	0.89
60	Glu 166	.	.	.		T	.	.	1.42	-0.47	.	F	.	1.88	1.15
	Gln 167	.	.	.		T	.	.	1.69	-0.43	.	F	.	1.82	1.32
	Cys 168	.	.	.		T	.	.	2.10	-0.31	.	F	.	1.76	1.34

**Table I (continued)**

	Res Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Gln 169	.	.	B	.	T	.	.	2.40	-0.70	.	.	F	1.94	1.52
	Pro 170	.	.	.	T	T	.	.	2.03	-0.30	.	.	F	2.32	1.41
	His 171	.	.	.	T	T	.	.	1.72	-0.13	.	.	F	2.80	1.41
10	Arg 172	.	.	.	T	T	.	.	1.13	-0.21	*	.	F	2.52	1.18
	Asn 173	.	.	.	T	T	.	.	0.99	-0.11	*	.	.	1.94	0.77
	Cys 174	.	.	B	.	T	.	.	0.64	0.14	.	.	.	0.66	0.47
	Thr 175	.	A	B	.	.	.	.	0.04	0.07	.	.	.	-0.02	0.24
	Ala 176	.	A	B	.	.	.	.	-0.51	0.76	*	.	.	-0.60	0.12
15	Leu 177	.	A	B	.	.	.	.	-1.43	0.86	*	.	.	-0.60	0.23
	Gly 178	.	A	B	.	.	.	.	-1.43	0.97	.	*	.	-0.60	0.13
	Leu 179	.	A	B	.	.	.	.	-1.62	0.89	.	*	.	-0.60	0.21
	Ala 180	.	A	B	.	.	.	.	-1.52	1.03	.	*	.	-0.60	0.19
	Leu 181	.	A	B	.	.	.	.	-1.28	0.77	.	*	.	-0.60	0.29
20	Asn 182	.	A	B	.	.	.	.	-0.77	0.77	*	.	.	-0.60	0.35
	Val 183	.	.	B	.	.	T	.	-0.72	0.47	*	.	F	-0.05	0.46
	Pro 184	.	.	.	.	T	T	C	-0.21	0.36	*	.	F	0.73	0.75
	Gly 185	.	.	.	T	T	.	.	0.34	0.06	.	*	F	1.21	0.63
	Ser 186	.	.	.	T	T	.	.	1.16	0.16	.	*	F	1.64	1.15
25	Ser 187	.	.	.	T	T	C	.	0.84	-0.49	.	.	F	2.32	1.24
	Ser 188	.	.	.	T	T	.	.	0.89	-0.43	.	.	F	2.80	1.81
	His 189	.	.	B	.	T	.	.	0.43	-0.17	.	.	F	2.12	1.11
	Asp 190	.	.	.	T	T	.	.	0.47	0.01	.	.	F	1.49	0.45
	Thr 191	.	.	B	.	.	.	.	0.47	0.11	.	.	F	0.61	0.48
30	Leu 192	.	.	B	.	.	.	.	0.10	0.11	.	.	.	0.18	0.47
	Cys 193	.	.	B	.	T	.	.	0.09	0.19	.	.	.	0.10	0.15
	Thr 194	.	.	B	.	T	.	.	-0.22	0.67	.	.	.	-0.20	0.15
	Ser 195	.	.	B	.	T	.	.	-0.92	0.61	*	.	F	-0.05	0.18
	Cys 196	.	.	B	.	T	.	.	-0.82	0.71	.	.	F	-0.05	0.29
35	Thr 197	.	.	.	T	.	.	.	-0.82	0.57	.	.	F	0.15	0.31
	Gly 198	.	.	.	T	.	.	.	-0.46	0.77	.	.	.	0.00	0.19
	Phe 199	.	.	B	.	.	.	.	-0.46	0.77	*	.	.	-0.40	0.48
	Pro 200	.	.	B	.	.	.	.	-0.04	0.69	*	*	.	-0.40	0.48
	Leu 201	.	.	B	.	.	.	.	-0.23	0.20	*	*	.	-0.10	0.96
40	Ser 202	.	.	B	.	.	.	.	-0.13	0.41	*	*	F	0.02	0.82
	Thr 203	.	.	B	.	.	.	.	-0.13	0.06	*	.	F	0.59	0.82
	Arg 204	.	.	.	.	.	C	.	-0.02	0.06	*	.	F	1.06	0.99
	Val 205	.	.	.	.	T	C	.	0.19	-0.13	*	.	F	2.13	0.74
	Pro 206	.	.	.	.	T	C	.	1.00	-0.51	*	.	F	2.70	0.89
45	Gly 207	.	.	.	.	T	C	.	0.63	-1.00	.	*	F	2.43	0.79
	Ala 208	A	.	.	.	T	.	.	0.94	-0.43	*	.	F	1.66	0.57
	Glu 209	A	A	.	.	.	.	.	0.94	-1.07	*	.	F	1.29	0.64
	Glu 210	A	A	.	.	.	.	.	1.21	-1.50	*	.	F	1.17	1.26
	Cys 211	A	A	.	.	.	.	.	0.57	-1.43	*	.	F	0.90	1.26
50	Glu 212	A	A	.	.	.	.	.	0.02	-1.29	*	*	F	0.75	0.54
	Arg 213	A	A	.	.	.	.	.	0.61	-0.60	*	*	.	0.60	0.22
	Ala 214	A	A	.	.	.	.	.	-0.09	-0.60	*	*	.	0.60	0.68
	Val 215	A	A	.	.	.	.	.	-0.94	-0.39	*	*	.	0.30	0.34
	Ile 216	A	A	.	.	.	.	.	-0.87	0.26	*	*	.	-0.30	0.13
55	Asp 217	A	A	.	.	.	.	.	-1.57	0.76	*	*	.	-0.60	0.13
	Phe 218	A	A	.	.	.	.	.	-1.68	1.04	*	*	.	-0.60	0.15
	Val 219	A	A	.	.	.	.	.	-1.09	0.80	.	.	.	-0.60	0.37
	Ala 220	A	A	.	.	.	.	.	-1.12	0.11	.	.	.	-0.30	0.37
	Phe 221	A	A	.	.	.	.	.	-0.53	0.80	*	.	.	-0.60	0.30
60	Gln 222	A	A	.	.	.	.	.	-1.42	0.40	*	.	.	-0.60	0.54
	Asp 223	A	A	.	.	.	.	.	-0.68	0.44	*	.	F	-0.45	0.38
	Ile 224	A	A	.	.	.	.	.	0.29	-0.06	.	.	F	0.45	0.87

**Table I (continued)**

	Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Ser	225	A	A	.	.	.	.	.	0.07	-0.84	.	.	F	0.75	0.99
	Ile	226	A	A	.	.	.	.	.	0.77	-0.56	*	*	F	0.75	0.49
	Lys	227	A	A	.	.	.	.	.	0.88	-0.16	*	*	F	0.60	1.20
	Arg	228	A	A	.	.	.	.	.	0.07	-0.84	*	*	F	0.90	1.76
10	Leu	229	A	A	.	.	.	.	.	0.14	-0.54	*	.	F	0.90	2.07
	Gln	230	A	A	.	.	.	.	.	0.44	-0.54	*	.	F	0.75	0.85
	Arg	231	.	A	B	.	.	.	.	0.74	-0.14	*	.	.	0.30	0.76
	Leu	232	A	A	.	.	.	.	.	-0.11	0.36	*	.	.	-0.30	0.93
15	Leu	233	.	A	B	.	.	.	.	-0.22	0.36	*	*	.	-0.30	0.44
	Gln	234	.	A	B	.	.	.	.	-0.00	-0.04	*	.	.	0.30	0.39
	Ala	235	.	A	B	.	.	.	.	-0.21	0.46	*	.	.	-0.60	0.48
	Leu	236	.	A	B	.	.	.	.	-0.32	0.20	*	*	.	-0.30	0.89
20	Glu	237	.	A	B	.	.	.	.	0.14	-0.49	.	.	.	0.30	0.89
	Ala	238	.	.	B	.	.	.	T	0.67	-0.46	.	.	F	0.85	0.88
	Pro	239	.	.	.	.	.	T	T	0.32	-0.04	.	.	F	1.40	1.12
	Glu	240	.	.	.	.	T	T	T	0.70	-0.30	.	.	F	1.25	0.64
25	Gly	241	.	.	.	.	T	T	T	1.20	0.13	.	.	F	0.65	0.98
	Trp	242	.	.	.	.	T	.	.	0.99	0.11	*	.	F	0.45	0.91
	Gly	243	.	.	.	.	.	.	C	1.69	0.11	*	*	F	0.59	0.81
	Pro	244	.	.	.	.	.	.	C	1.31	0.11	*	*	F	1.08	1.61
30	Thr	245	.	.	.	.	.	T	C	0.97	0.19	*	.	F	1.62	1.55
	Pro	246	.	.	.	.	.	T	C	1.42	-0.30	*	.	F	2.56	1.55
	Arg	247	.	.	.	.	T	T	C	1.12	-0.73	*	.	F	3.40	1.96
	Ala	248	.	.	.	.	.	T	C	0.88	-0.66	*	.	F	2.86	1.37
35	Gly	249	A	A	.	.	.	.	.	0.28	-0.64	*	*	F	1.77	0.90
	Arg	250	A	A	.	.	.	.	.	0.59	-0.39	*	*	.	0.98	0.38
	Ala	251	A	A	.	.	.	.	.	-0.01	0.01	*	*	.	0.04	0.65
	Ala	252	A	A	.	.	.	.	.	-0.08	0.20	*	*	.	-0.30	0.54
40	Leu	253	A	A	.	.	.	.	.	-0.30	-0.23	*	*	.	0.30	0.55
	Gln	254	A	A	.	.	.	.	.	0.16	0.46	.	*	.	-0.60	0.45
	Leu	255	A	A	.	.	.	.	.	0.16	-0.04	.	*	.	0.30	0.87
	Lys	256	A	A	.	.	.	.	.	0.86	-0.54	.	*	.	0.75	2.07
45	Leu	257	A	A	.	.	.	.	.	0.63	-1.23	.	*	F	0.90	2.34
	Arg	258	A	A	.	.	.	.	.	1.13	-0.94	*	*	F	0.90	2.34
	Arg	259	.	A	B	.	.	.	.	1.13	-1.14	*	*	F	0.90	1.69
	Arg	260	.	A	B	.	.	.	.	1.13	-1.14	*	*	F	0.90	3.55
50	Leu	261	.	A	B	.	.	.	.	0.28	-1.14	*	*	F	0.90	1.49
	Thr	262	.	A	B	.	.	.	.	0.74	-0.46	*	*	F	0.45	0.63
	Glu	263	.	A	B	.	.	.	.	0.04	-0.03	*	*	.	0.30	0.32
	Leu	264	.	A	B	.	.	.	.	-0.07	0.47	*	.	.	-0.60	0.39
55	Leu	265	.	A	B	.	.	.	.	-0.18	0.19	.	*	.	-0.30	0.47
	Gly	266	A	A	.	.	.	T	.	0.29	-0.30	.	.	.	0.30	0.45
	Ala	267	A	A	.	.	.	T	.	0.01	0.13	.	.	F	0.25	0.54
	Gln	268	A	.	.	.	T	.	-0.80	-0.06	.	.	F	0.85	0.66	
60	Asp	269	A	.	.	.	T	.	-0.80	-0.06	.	.	F	0.85	0.55	
	Gly	270	A	.	.	.	T	.	-0.84	0.20	*	*	.	0.10	0.45	
	Ala	271	A	A	.	.	.	.	-0.39	0.34	*	*	.	-0.30	0.19	
	Leu	272	.	A	B	.	.	.	-0.61	-0.06	*	*	.	0.30	0.23	
65	Leu	273	.	A	B	.	.	.	-1.42	0.63	*	*	.	-0.60	0.19	
	Val	274	A	A	.	.	.	.	-1.42	0.89	*	*	.	-0.60	0.15	
	Arg	275	A	A	.	.	.	.	-1.67	0.79	*	*	.	-0.60	0.32	
	Leu	276	A	A	.	.	.	.	-1.89	0.60	*	*	.	-0.60	0.40	
70	Leu	277	A	A	.	.	.	.	-0.97	0.60	*	*	.	-0.60	0.44	
	Gln	278	A	A	.	.	.	.	-1.01	-0.04	*	*	.	0.30	0.44	
	Ala	279	A	A	.	.	.	.	-0.74	0.60	*	*	.	-0.60	0.40	
	Leu	280	A	A	.	.	.	.	-0.74	0.41	*	*	.	-0.60	0.49	

**Table I (continued)**

	Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Arg	281	.	A	B	.	.	.	.	-0.53	-0.27	*	.	.	0.30	0.55
	Val	282	.	A	B	.	.	.	.	0.07	-0.06	*	.	.	0.30	0.54
	Ala	283	.	A	B	.	.	.	.	-0.28	-0.13	*	.	.	0.72	1.01
10	Arg	284	.	A	B	.	.	.	.	-0.50	-0.39	*	.	.	0.84	0.51
	Met	285	.	.	B	.	.	T	.	0.31	0.30	.	*	.	0.91	0.57
	Pro	286	.	.	.	.	T	C	0.31	-0.34	.	*	F	2.13	0.97	
	Gly	287	.	.	.	.	T	C	0.87	-0.84	*	*	F	2.70	0.97	
15	Leu	288	A	.	.	.	.	T	.	0.60	-0.46	*	*	F	2.08	1.32
	Glu	289	A	.	.	.	.	.	.	0.60	-0.43	*	*	F	1.46	0.63
	Arg	290	A	.	.	.	.	.	.	1.20	-0.86	*	*	F	1.64	1.25
	Ser	291	A	.	.	.	.	.	.	1.52	-1.29	*	*	F	1.37	2.62
	Val	292	A	.	.	.	.	.	.	1.17	-1.97	*	*	F	1.10	2.97
	Arg	293	A	.	.	.	.	.	.	1.17	-1.19	*	*	F	1.10	1.31
20	Glu	294	A	.	.	.	.	.	.	0.96	-0.50	*	*	F	0.65	0.81
	Arg	295	A	.	.	.	.	.	.	-0.01	-0.46	*	*	F	0.80	1.68
	Phe	296	.	.	B	.	.	.	.	0.26	-0.46	.	*	.	0.50	0.64
	Leu	297	.	.	B	.	.	.	.	0.72	0.04	.	*	.	-0.10	0.50
	Pro	298	A	.	.	.	.	.	.	0.22	0.47	.	*	.	-0.40	0.33
25	Val	299	A	.	.	.	.	.	.	-0.17	0.90	*	.	.	-0.40	0.48
	His	300	A	.	.	.	.	.	.	-0.67	0.54	.	.	.	-0.40	0.75

**Table II**

	Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Met	1	.	.	B	.	.	.	.	0.06	0.09	*	.	-0.10	0.60	
	Arg	2	.	.	B	.	.	.	.	0.10	-0.34	*	.	0.50	0.82	
	Ala	3	.	.	B	.	.	.	.	0.28	-0.34	*	.	0.50	0.63	
	Leu	4	.	.	B	.	.	.	.	0.32	-0.34	.	.	0.50	0.99	
10	Glu	5	.	.	B	.	.	.	.	-0.10	-0.53	.	F	0.95	0.50	
	Gly	6	.	.	.	.	.	T	C	0.20	0.16	*	F	0.45	0.41	
	Pro	7	.	.	.	.	T	T	.	-0.72	0.04	*	F	0.65	0.66	
	Gly	8	.	.	.	.	T	T	.	-0.94	0.04	.	F	0.65	0.32	
	Leu	9	.	.	B	.	.	T	.	-0.80	0.73	.	.	-0.20	0.26	
15	Ser	10	.	A	B	.	.	.	.	-1.61	0.87	.	.	-0.60	0.09	
	Leu	11	.	A	B	.	.	.	.	-2.12	1.13	.	.	-0.60	0.08	
	Leu	12	.	A	B	.	.	.	.	-2.72	1.34	.	.	-0.60	0.07	
	Cys	13	.	A	B	.	.	.	.	-2.97	1.34	.	.	-0.60	0.04	
	Leu	14	.	A	B	.	.	.	.	-2.97	1.46	.	.	-0.60	0.05	
20	Val	15	.	A	B	.	.	.	.	-2.88	1.46	.	.	-0.60	0.05	
	Leu	16	.	A	B	.	.	.	.	-2.66	1.20	.	.	-0.60	0.15	
	Ala	17	.	A	B	.	.	.	.	-2.66	1.13	.	.	-0.60	0.18	
	Leu	18	.	A	B	.	.	.	.	-2.80	1.13	.	.	-0.60	0.20	
	Pro	19	.	A	B	.	.	.	.	-2.20	1.17	.	.	-0.60	0.20	
25	Ala	20	.	A	B	.	.	.	.	-2.20	0.91	.	.	-0.60	0.31	
	Leu	21	.	A	B	.	.	.	.	-1.60	1.06	*	.	-0.60	0.28	
	Leu	22	.	A	B	.	.	.	.	-1.60	0.80	.	.	-0.60	0.28	
	Pro	23	.	A	B	.	.	.	.	-1.64	0.87	*	.	-0.60	0.28	
	Val	24	.	B	.	.	.	.	.	-1.32	1.01	*	.	-0.40	0.25	
	Pro	25	.	B	.	.	.	.	.	-1.08	0.33	.	.	-0.10	0.60	
30	Ala	26	.	B	B	.	.	.	.	-1.12	0.07	.	.	-0.30	0.38	
	Val	27	.	B	B	.	.	.	.	-0.90	0.29	*	*	-0.30	0.38	
	Arg	28	.	B	B	.	.	.	.	-0.69	0.14	*	*	-0.30	0.25	
	Gly	29	.	B	B	.	.	.	.	-0.14	-0.29	*	*	0.30	0.43	
35	Val	30	.	B	B	.	.	.	.	-0.14	-0.30	*	*	0.30	0.83	
	Ala	31	.	B	B	.	.	.	.	0.13	-0.51	*	*	0.60	0.66	
	Glu	32	.	B	.	.	.	.	T	0.74	-0.03	*	*	F	0.65	0.96
	Thr	33	.	B	.	.	.	.	T	0.42	0.30	*	*	F	0.40	2.02
	Pro	34	.	.	.	.	T	T	.	0.48	0.09	*	*	F	0.80	3.10
40	Thr	35	.	.	.	.	T	T	.	1.44	0.50	*	.	F	0.50	1.88
	Tyr	36	.	.	.	.	.	T	C	2.03	0.50	*	.	0.15	2.55	
	Pro	37	.	.	.	.	T	.	.	1.44	0.01	*	.	0.45	2.76	
	Trp	38	.	A	.	.	.	.	C	1.76	0.09	*	.	0.05	1.93	
	Arg	39	.	A	B	.	.	.	.	1.66	-0.40	*	.	F	0.60	2.13
45	Asp	40	.	A	.	.	.	.	C	1.62	-0.67	*	.	F	1.10	1.99
	Ala	41	.	A	.	.	.	.	C	1.87	-0.67	*	*	F	1.10	1.87
	Glu	42	.	A	.	.	T	.	C	2.19	-1.59	*	*	F	1.10	1.66
	Thr	43	.	A	.	.	T	.	.	1.67	-1.59	*	.	F	1.30	1.94
	Gly	44	.	A	.	.	T	.	.	0.70	-0.90	*	.	F	1.30	1.59
50	Glu	45	.	A	.	.	T	.	.	0.03	-0.76	*	*	F	1.15	0.68
	Arg	46	.	A	.	.	T	.	.	0.03	-0.19	*	.	F	0.85	0.25
	Leu	47	.	A	B	.	.	.	.	0.03	-0.17	*	.	0.30	0.26	
	Val	48	.	A	B	.	.	.	.	-0.32	-0.20	.	.	0.30	0.26	
	Cys	49	.	A	B	.	.	.	.	-0.19	0.37	.	.	-0.30	0.07	
	Ala	50	.	A	B	.	.	.	.	-0.40	0.80	.	.	-0.60	0.13	
55	Gln	51	.	A	B	.	.	.	.	-0.86	0.54	.	.	-0.60	0.28	
	Cys	52	.	A	B	.	.	.	.	-0.36	0.33	.	.	-0.30	0.51	
	Pro	53	.	.	.	.	T	T	C	-0.20	0.24	.	.	F	0.45	0.73
	Pro	54	.	.	.	.	T	T	.	-0.39	0.53	.	.	F	0.35	0.36
	Gly	55	.	.	.	.	T	T	.	0.20	0.77	*	.	F	0.35	0.50
60	Thr	56	.	.	B	.	.	T	.	0.31	0.60	.	.	F	-0.05	0.56

**Table II (continued)**

	Res Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	
5	Phe 57	.	.	B	B	.	.	.	0.77	0.17	*	.	F	-0.15	0.71	
	Val 58	.	.	B	B	.	.	.	0.31	0.17	*	.	.	0.19	1.12	
	Gln 59	.	.	B	B	.	.	.	0.63	0.31	*	.	F	0.53	0.41	
10	Arg 60	.	.	B	.	T	.	.	1.09	-0.17	*	.	F	1.87	0.94	
	Pro 61	.	.	B	.	T	.	.	1.40	-0.96	*	.	F	2.66	2.47	
	Cys 62	.	.	.	.	T	T	.	1.80	-1.60	*	.	F	3.40	2.38	
	Arg 63	.	.	.	.	T	T	.	2.44	-1.61	*	.	F	3.06	1.63	
	Arg 64	.	.	.	.	T	.	.	2.13	-1.19	*	.	F	2.77	1.63	
15	Asp 65	.	.	.	.	T	.	.	1.71	-1.13	*	.	F	2.68	4.39	
	Ser 66	.	.	.	.	T	T	.	1.26	-1.21	.	.	F	2.79	3.24	
	Pro 67	.	.	.	.	T	T	.	1.58	-0.64	.	.	F	2.55	0.89	
	Thr 68	.	.	.	.	T	T	.	1.26	-0.21	.	.	F	2.50	0.52	
	Thr 69	.	.	.	.	T	T	.	0.48	0.21	.	.	F	1.65	0.61	
20	Cys 70	.	.	.	.	T	.	.	0.27	0.40	.	.	F	1.14	0.21	
	Gly 71	.	.	.	.	T	T	.	0.36	0.40	.	*	F	1.33	0.22	
	Pro 72	.	.	.	.	T	T	.	0.68	0.34	.	*	F	1.62	0.24	
	Cys 73	.	.	.	.	.	T	C	0.96	-0.14	*	.	F	2.01	0.88	
	Pro 74	.	.	.	.	.	T	C	1.02	-0.21	*	.	F	2.40	1.21	
25	Pro 75	.	.	.	.	T	T	.	1.38	0.11	*	*	F	1.76	1.23	
	Arg 76	.	.	.	.	T	T	.	1.72	0.17	*	*	F	1.52	3.30	
	His 77	.	.	B	.	.	T	.	1.23	0.00	*	*	F	0.88	3.70	
	Tyr 78	.	.	B	.	.	T	.	1.61	0.36	*	*	.	0.49	2.07	
	Thr 79	.	.	B	.	.	.	.	1.82	0.84	*	*	.	-0.25	1.11	
30	Gln 80	.	.	B	.	.	.	.	1.79	1.24	*	*	.	-0.25	1.31	
	Phe 81	.	.	.	.	T	.	.	0.87	1.50	*	*	.	0.15	1.31	
	Trp 82	.	.	.	.	T	.	.	0.90	1.43	*	.	.	0.00	0.75	
	Asn 83	.	A	.	.	T	.	.	1.26	0.94	*	.	.	-0.20	0.75	
	Tyr 84	.	A	.	.	T	.	.	0.90	0.54	*	*	.	-0.05	1.70	
35	Leu 85	.	A	.	.	T	.	.	1.01	0.33	*	*	.	0.38	0.87	
	Glu 86	.	A	.	.	T	.	.	1.47	-0.59	*	*	.	1.71	1.05	
	Arg 87	.	A	.	.	T	.	.	1.09	-0.23	.	.	.	1.69	1.05	
	Cys 88	.	.	.	.	T	T	.	1.09	-0.41	.	*	.	2.22	0.69	
	Arg 89	.	.	.	.	T	T	.	0.48	-0.70	.	*	.	2.80	0.64	
40	Tyr 90	.	.	.	.	T	T	.	0.48	-0.06	.	*	.	2.22	0.24	
	Cys 91	.	.	.	.	T	T	.	-0.19	0.63	.	*	.	1.04	0.37	
	Asn 92	.	.	B	B	.	.	.	-0.64	0.63	.	*	.	-0.04	0.10	
	Val 93	.	.	B	B	.	.	.	0.02	1.06	.	*	.	-0.02	0.06	
	Leu 94	.	.	B	B	.	.	.	0.02	0.30	.	.	.	0.30	0.21	
45	Cys 95	.	.	B	.	.	T	C	0.27	-0.27	.	.	.	1.60	0.25	
	Gly 96	.	.	.	.	T	C	0.93	-0.67	.	.	.	F	2.55	0.59	
	Glu 97	.	.	.	.	T	C	0.93	-1.31	.	.	.	F	3.00	1.24	
	Arg 98	A	.	.	.	T	.	.	1.20	-2.00	.	*	.	F	2.50	4.00
	Glu 99	A	A	.	.	.	.	.	2.12	-2.07	.	*	.	F	1.80	4.08
50	Glu 100	A	A	A	.	.	.	.	2.20	-2.50	.	*	.	F	1.50	4.61
	Glu 101	A	A	A	.	.	.	.	1.88	-2.00	.	*	.	F	1.20	2.38
	Ala 102	A	A	A	.	.	.	.	1.84	-1.43	.	.	.	F	0.75	0.74
	Arg 103	A	A	A	.	.	.	.	1.14	-0.93	.	.	.	0.60	0.58	
	Ala 104	A	A	A	.	.	.	.	0.83	-0.43	.	*	.	0.30	0.34	
55	Cys 105	A	A	A	.	.	.	.	0.80	0.06	.	*	.	-0.30	0.48	
	His 106	A	A	A	.	.	.	.	0.80	0.06	*	*	.	-0.30	0.34	
	Ala 107	.	A	A	.	.	T	.	1.50	0.46	*	*	.	-0.20	0.53	
	Thr 108	.	A	.	.	T	.	.	0.80	-0.04	*	*	.	0.85	1.95	
	His 109	.	A	.	.	T	.	.	0.72	-0.11	*	.	.	1.13	1.45	
60	Asn 110	.	A	.	.	T	.	.	1.50	-0.04	*	.	.	1.26	0.77	
	Arg 111	.	A	.	.	T	.	.	0.87	-0.54	.	*	.	1.99	1.04	
	Ala 112	.	A	.	.	T	.	.	1.57	-0.46	.	*	.	1.82	0.41	

**Table II (continued)**

	Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
5	Cys	113	.	.	.		T	T	.	1.57	-0.96	.	*	.	2.80	0.50
	Arg	114	.	.	B	.	T	T	.	1.26	-0.87	.	*	.	2.12	0.37
	Cys	115	.	.	.		T	T	.	0.56	-0.44	*	*	.	1.94	0.36
	Arg	116	.	.	.		T	T	.	-0.26	-0.16	.	*	.	1.66	0.58
10	Thr	117	.	A	.	B	T	.	.	-0.26	0.06	.	*	F	0.53	0.26
	Gly	118	.	A	B	B	T	.	.	0.38	0.56	.	*	.	-0.20	0.49
	Phe	119	.	A	B	B	.	.	.	-0.32	0.49	.	*	.	-0.60	0.34
	Phe	120	.	A	B	B	.	.	.	-0.00	0.99	.	*	.	-0.60	0.24
	Ala	121	.	A	B	B	.	.	.	-0.81	0.93	.	*	.	-0.60	0.24
15	His	122	.	A	.	.	.	.	C	-1.17	1.29	.	*	.	-0.40	0.24
	Ala	123	.	A	.	.	.	.	C	-1.63	1.07	.	*	.	-0.40	0.15
	Gly	124	.	A	.	.	T	.	.	-0.93	0.97	.	*	.	-0.20	0.12
	Phe	125	.	A	.	.	T	.	.	-0.27	0.47	.	*	.	-0.20	0.15
	Cys	126	.	A	.	.	T	.	.	-0.27	0.47	.	*	.	-0.20	0.20
20	Leu	127	.	A	B	.	.	.	.	-0.53	0.47	.	*	.	-0.60	0.21
	Glu	128	.	A	B	.	T	.	.	-0.61	0.43	.	*	.	-0.20	0.32
	His	129	.	.	.	.	T	T	.	-0.48	0.21	.	.	.	0.50	0.32
	Ala	130	.	.	.	.	T	T	.	0.01	0.07	.	.	.	0.63	0.61
	Ser	131	.	.	.	.	T	T	.	0.33	-0.19	.	.	.	1.36	0.54
25	Cys	132	.	.	.	.	.	T	C	0.56	0.24	.	.	.	0.69	0.39
	Pro	133	.	.	.	.	.	T	C	0.21	0.24	.	.	F	0.97	0.39
	Pro	134	.	.	.	.	T	T	.	-0.61	0.17	.	.	F	1.30	0.29
	Gly	135	.	.	.	.	T	T	.	-0.91	0.43	.	.	F	0.87	0.40
	Ala	136	.	.	B	.	.	T	.	-1.20	0.54	.	.	.	0.19	0.18
30	Gly	137	.	.	B	B	.	.	.	-0.74	0.61	.	.	.	-0.34	0.12
	Val	138	.	.	B	B	.	.	.	-0.88	0.61	.	.	.	-0.47	0.19
	Ile	139	.	.	B	B	.	.	.	-0.67	0.61	.	.	.	-0.60	0.18
	Ala	140	.	.	B	.	.	T	.	-0.62	0.11	.	.	.	0.10	0.32
	Pro	141	.	.	B	.	.	T	.	-0.32	0.07	.	.	F	0.25	0.58
35	Gly	142	.	.	.	.	.	T	C	-0.57	0.34	*	*	F	0.45	0.86
	Glu	143	.	.	.	.	.	T	C	0.40	0.16	*	*	F	0.45	0.86
	Ser	144	.	.	B	.	.	.	.	0.94	-0.34	*	*	F	0.80	1.10
	Trp	145	.	.	.	.	T	.	.	1.19	-0.34	*	*	F	1.20	1.10
	Ala	146	.	.	B	.	.	T	.	0.81	-0.34	*	*	F	0.85	0.63
40	Arg	147	.	.	.	.	T	T	.	0.94	0.16	*	*	F	0.65	0.47
	Gly	148	.	.	.	.	T	T	.	1.06	0.20	.	*	F	0.65	0.69
	Gly	149	.	.	.	.	.	T	C	1.06	-0.71	.	.	F	1.84	1.35
	Ala	150	.	.	.	.	.	.	C	1.00	-0.83	.	.	F	1.83	0.92
	Pro	151	.	.	.	.	.	.	C	1.24	-0.40	.	*	F	1.87	0.92
45	Arg	152	.	.	.	.	T	T	.	1.24	-0.40	.	.	F	2.61	0.92
	Ser	153	.	.	.	.	T	T	.	1.70	-0.83	*	.	F	3.40	1.78
	Gly	154	.	.	.	.	T	T	.	1.38	-1.33	*	*	F	3.06	2.26
	Gly	155	.	.	.	.	T	T	.	1.62	-1.19	*	*	F	2.57	0.62
	Arg	156	.	.	.	.	T	.	.	1.94	-0.76	*	*	F	2.26	0.46
50	Arg	157	.	.	.	.	T	.	.	1.49	-1.14	*	*	F	2.15	0.90
	Cys	158	.	.	B	.	.	.	.	1.79	-1.14	*	*	F	1.64	0.90
	Gly	159	.	.	.	.	T	T	.	1.28	-1.17	*	*	F	2.47	0.80
	Arg	160	.	.	B	.	.	T	.	1.03	-0.53	*	*	F	2.30	0.30
	Gly	161	.	.	B	.	.	T	.	0.58	-0.03	*	*	F	1.77	0.57
	Gln	162	.	.	B	.	.	T	.	0.26	-0.17	*	*	F	1.54	0.57
55	Val	163	.	.	B	.	.	.	.	0.62	-0.17	*	*	F	1.11	0.45
	Ala	164	.	.	B	.	.	.	.	0.16	0.21	.	*	F	0.28	0.61
	Gly	165	.	.	B	.	.	T	.	-0.54	0.47	.	*	F	-0.05	0.29
	Pro	166	.	.	B	.	.	T	.	-0.41	0.57	.	.	F	-0.05	0.40
60	Ser	167	.	.	.	.	.	T	C	-0.80	0.36	.	.	F	0.45	0.61
	Leu	168	.	.	B	.	.	T	.	-0.33	0.29	.	.	.	0.10	0.78
	Ala	169	.	.	B	.	.	.	.	-0.13	0.29	.	.	.	-0.10	0.65
	Pro	170	.	.	B	.	.	.	.	-0.18	0.29	.	.	.	-0.10	0.62

Additional preferred nucleic acid fragments of the present invention comprise, or alternatively consist of, nucleic acid molecules encoding one or more epitope-bearing portions of TNFR-6 $\alpha$  and/or TNFR-6 $\beta$ . In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising, or alternatively consisting of, amino acid residues from about Phe-57 to about Thr-117, from about Cys-132 to about Thr-175, from about Gly-185 to about Thr-194, from about Val-205 to about Asp-217, from about Pro-239 to about Leu-264, and/or from about Ala-283 to about Pro-298 in SEQ ID NO:2. In additional embodiments, nucleic acid fragments of the present invention comprise, or alternatively consist of nucleic acid molecules encoding one or more epitope bearing portions of TNFR-6 $\beta$  from about Ala-31 to about Thr-46, from about Phe-57 to about Gln-80, from about Glu-86 to about His-106, from about Thr-108 to about Phe-119, from about His-129 to about Val-138, and/or from about Gly-142 to about Pro-166 in SEQ ID NO:4. In this context “about” includes the particularly recited ranges and ranges larger or smaller by several (5, 4, 3, 2, or 1) amino acids at either terminus or at both termini. These polypeptide fragments have been determined to bear antigenic epitopes of the TNFR-6 $\alpha$  and TNFR-6 $\beta$  polypeptides respectively, by the analysis of the Jameson-Wolf antigenic index, as shown in Figures 4 and 5, above. Further, polypeptide fragments which bear antigenic epitopes of TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  may be easily determined by one of skill in the art using the above-described analysis of the Jameson-Wolf antigenic index, as shown in Figures 4 and 5. Methods for determining other such epitope-bearing portions of TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  are described in detail below.

In specific embodiments, the nucleic acids of the invention are less than 100000 kb, 50000 kb, 10000 kb, 1000 kb, 500 kb, 400 kb, 350 kb, 300 kb, 250

kb, 200 kb, 175 kb, 150 kb, 125 kb, 100 kb, 75 kb, 50 kb, 40 kb, 30 kb, 25 kb, 20 kb, 15 kb, 10 kb, 7.5 kb, or 5 kb in length.

In further embodiments, nucleic acids of the invention comprise at least 15, at least 30, at least 50, at least 100, or at least 250, at least 500, or at least 1000 contiguous nucleotides of TNFR coding sequence, but consist of less than or equal to 1000 kb, 500 kb, 250 kb, 200 kb, 150 kb, 100 kb, 75 kb, 50 kb, 30 kb, 25 kb, 20 kb, 15 kb, 10 kb, or 5 kb of genomic DNA that flanks the 5' or 3' coding nucleotide sequence set forth in Figure 1 (SEQ ID NO:1) or

<sup>a</sup>  
Figures 2A-B  
<sup>a</sup>  
Figure 2 (SEQ ID NO:3). In further embodiments, nucleic acids of the

invention comprise at least 15, at least 30, at least 50, at least 100, or at least 250, at least 500, or at least 1000 contiguous nucleotides of TNFR coding sequence, but do not comprise all or a portion of any TNFR intron. In another embodiment, the nucleic acid comprising TNFR coding sequence does not contain coding sequences of a genomic flanking gene (i.e., 5' or 3' to the TNFR gene in the genome). In other embodiments, the nucleic acids of the invention do not contain the coding sequence of more than 1000, 500, 250, 100, 50, 25, 20, 15, 10, 5, 4, 3, 2, or 1 genomic flanking gene(s).

In another aspect, the invention provides an isolated nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809, or a fragment of the polynucleotide sequence disclosed in

<sup>a</sup>  
Figures 2A-B  
Figure 1 and/or Figure 2. By "stringent hybridization conditions" is intended overnight incubation at 42° C in a solution comprising: 50% formamide, 5x SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65° C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 (e.g., 50) nt of the reference polynucleotide. These have uses that include, but are not limited to, as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least about 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., a deposited cDNA or a nucleotide sequence as shown in Figure 1 or <sup>Figures 2A-B</sup> SEQ ID NO:1 or 3)). In this context "about" includes the particularly recited size, and those sizes that are larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini. Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of a TNFR cDNA, or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone that has been generated using oligo dT as a primer).

As indicated, nucleic acid molecules of the present invention which encode a TNFR polypeptide may include, but are not limited to, those encoding the amino acid sequence of the mature polypeptide, by itself; and the coding sequence for the mature polypeptide and additional sequences, such as those encoding the about 26-35 amino acid leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences.

Also encoded by nucleic acids of the invention are the above protein sequences together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; an additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities.

Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37: 767 (1984). As discussed below, other such fusion proteins include a TNFR-6 $\alpha$  or TNFR-6 $\beta$  fused to Fc at the N- or C-terminus.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of a TNFR polypeptide. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques which include, but are not limited to oligonucleotide

mediated mutagenesis, alanine scanning, PCR mutagenesis, site directed mutagenesis (see e.g., Carter et al., *Nucl. Acids Res.* 13:4331 (1986); and Zoller et al., *Nucl. Acids Res.* 10:6487 (1982)), cassette mutagenesis (see e.g., Wells et al., *Gene* 34:315 (1985)), restriction selection mutagenesis (see e.g., Wells et al., *Philos. Trans. R. Soc. London SerA* 317:415 (1986)).

Thus, the invention also encompasses TNFR variants (e.g., derivatives and analogs) that have one or more amino acid residues deleted, added, or substituted to generate TNFR polypeptides that are better suited for expression, scale up, etc., in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges; N-linked glycosylation sites can be altered or eliminated to achieve, for example, expression of a homogeneous product that is more easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety of amino acid substitutions at one or both of the first or third amino acid positions on any one or more of the glycosylation recognition sequences in the TNFR polypeptides of the invention, and/or an amino acid deletion at the second position of any one or more such recognition sequences will prevent glycosylation of the TNFR at the modified tripeptide sequence (see, e.g., Miyajima et al., *EMBO J* 5(6):1193-97). Additionally, one or more of the amino acid residues of the polypeptides of the invention (e.g., arginine and lysine residues) may be deleted or substituted with another residue to eliminate undesired processing by proteases such as, for example, furins or kexins. For example, polypeptides of the invention containing carboxy terminal TNFR polypeptide sequences may have the amino acid residue corresponding to the arginine residue at position 290 and/or 295 of SEQ ID NO:2 deleted or substituted with another residue.

Variants of the invention include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions

may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the TNFR polypeptide or portions thereof. Also especially preferred in this regard are conservative substitutions.

Highly preferred are nucleic acid molecules encoding a mature protein having an amino acid sequence shown in SEQ ID NOS:2 and 4 or the mature TNFR polypeptide sequences encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or ATCC Deposit No. 97809.

Further embodiments include an isolated nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 80%, 85%, 90%, 92%, or 95%, 96%, 97%, 98% or 99% identical to a polynucleotide selected from the group consisting of: (a) a nucleotide sequence encoding a TNFR polypeptide having the complete amino acid sequence in SEQ ID NO:2 or 4, or as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809; (b) a nucleotide sequence encoding a mature TNFR polypeptide having an amino acid sequence at positions 31-300 or 31-170 in SEQ ID NO:2 or 4, respectively, or as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809; (c) a nucleotide sequence encoding a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31-283 and 31-166 of SEQ ID NOS:2 and 4, respectively; (d) a nucleotide sequence encoding a fragment of the TNFR polypeptide having the complete amino acid sequence in SEQ ID NO:2 or 4, or as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809, wherein the

fragment has TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  functional activity; and (e) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c) or (d) above. Polypeptides encoded by the polynucleotides are also encompassed by the invention.

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 80%, 85%, 90%, **92%**, or 95%, 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences in (a), (b), (c), (d), or (e), above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide in (a), (b), (c), (d), or (e), above. This polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues. An additional nucleic acid embodiment of the invention relates to an isolated nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide having an amino acid sequence in (a), (b), (c), (d), or (e), above.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a TNFR polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the TNFR polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 80%, 85%, 90%, **92%**, or 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3'

terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The reference sequence may be the entire TNFR-6 $\alpha$  and/or TNFR -6 $\beta$  encoding sequence shown in Figures 1 (SEQ ID NO:1 and 2) <sup>Figures 2A-B</sup> and <sup>Figures 2A-B</sup> Figure 2 (SEQ ID NO:3 and 4) or any fragment, variant, derivative or analog thereof, as described herein.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, a nucleotide sequence shown in Figure 1 or <sup>Figures 2A-B</sup> <sup>Figures 2A-B</sup> or to the nucleotides sequence contained in one or both of the deposited cDNA clones can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed. The reference (query) sequence may be the entire TNFR encoding nucleotide sequence shown in Figure 1 (SEQ ID NO:1), <sup>Figure 5</sup> <sup>Figures 2A-B</sup> <sup>Figures 2A-B</sup> Figure 2 (SEQ ID NO:3) or any TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  polynucleotide fragment (e.g., a polynucleotide encoding the amino acid sequence of any of the N or C terminal deletions described herein), variant, derivative or analog, as described herein.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (*Comp. App. Biosci.* 6:237-245 (1990)). Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. A determination of whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of this embodiment. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score. For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at

5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in Figures 2A-B, 15 in Figure 1 or 2 (SEQ ID NO:1 or 3), to the nucleic acid sequence of a deposited cDNA and/or to a nucleic acid sequence otherwise disclosed herein (e.g., encoding polypeptide having the amino acid sequence of a N and/or C terminal deletion disclosed herein, such as, for example, a nucleic acid molecule encoding amino acids Val-30 to His-300 of SEQ ID NO:2), irrespective of whether they encode a polypeptide having TNFR functional activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having TNFR functional activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having TNFR functional activity include, inter alia, (1) isolating a TNFR gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the TNFR gene, as described in Verma *et al.*, *Human*

*Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and Northern Blot analysis for detecting TNFR mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 5 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown figures 2A-B in Figure 1 or <sup>figures 2A-B</sup> (SEQ ID NOS:1 or 3) or to the nucleic acid sequence of the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or ATCC Deposit No. 97809, and/or to a nucleic acid sequence otherwise disclosed herein (e.g., encoding polypeptide having the amino acid sequence of a N and/or C terminal deletion disclosed herein), which do, in fact, encode 10 polypeptides having TNFR (i.e., TNFR-6 $\alpha$  and/or TNFR-6 $\beta$ ) protein functional activity. By "a polypeptide having TNFR functional activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of a TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  protein of the invention (e.g., 15 complete (full-length), mature, and extracellular domain as measured, for example, in a particular immunoassay or biological assay. For example, TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  activity can be measured by determining the ability of a TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  polypeptide to bind a TNFR-6 $\alpha$  and/or -6 $\beta$  ligand (e.g., Fas Ligand and/or AIM-II (International application 20 publication number WO 97/34911, published September 25, 1997). In another example, TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  functional activity is measured by determining the ability of a polypeptide, such as cognate ligand which is free or expressed on a cell surface, to induce apoptosis.

The TNF family ligands induce various cellular responses by binding to 25 TNF-family receptors, including the TNFR-6 $\alpha$  and TNFR-6 $\beta$  of the present invention. Cells which express the TNFR proteins are believed to have a potent cellular response to TNFR-I receptor ligands including B lymphocytes (CD19+), both CD4 and CD8+ T lymphocytes, monocytes and endothelial

cells. By a "cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphological change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased cell proliferation or the inhibition of increased cell proliferation, such as by the inhibition of apoptosis.

Screening assays for the forgoing are known in the art. One such screening assay involves the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in Science 246:181-296 (October 1989). For example, a TNF-family ligand may be contacted with a cell which expresses the mature form of the receptor polypeptide of the present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the TNFR polypeptide is active.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the cDNA clone deposited as ATCC Deposit No. 97810 or 97809, the nucleic acid sequence shown in Figure 1 or <sup>Figures 2A-B</sup> (SEQ ID NO:1 and 3), or fragments thereof, will encode a polypeptide "having TNFR protein functional activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having TNFR protein functional activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less

likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid), as further described below.

**5        Vectors and Host Cells**

The present invention also relates to vectors which include the isolated nucleic acid molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, or which are otherwise engineered to produce the polypeptides of the invention, and the production of TNFR polypeptides, or fragments thereof, by recombinant techniques.

In one embodiment, the polynucleotides of the invention are joined to a vector (e.g., a cloning or expression vector). The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs

will preferably include a translation initiating codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium.

Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, for example, stabilization or simplified purification of expressed recombinant product.

In one embodiment, the DNA of the invention is operatively associated with an appropriate heterologous regulatory element (e.g., promoter or enhancer), such as, the phage lambda PL promoter, the *E. coli lac, trp, phoA*, and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria.

Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila S2* and *Spodoptera Sf9* cells; animal cells such as CHO, COS, 293 and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

The host cell can be a higher eukaryotic cell, such as a mammalian cell (e.g., a human derived cell), or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. The host strain

may be chosen which modulates the expression of the inserted gene sequences, or modifies and processes the gene product in the specific fashion desired.

Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically engineered polypeptide may be controlled. Furthermore, different host cells have characteristics and specific mechanisms for the translational and post-translational processing and modification (e.g., phosphorylation, cleavage) of proteins. Appropriate cell lines can be chosen to ensure the desired modifications and processing of the foreign protein expressed. Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice. As a representative, but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. Among vectors

preferred for use in bacteria include pHE4-5 (ATCC Accession No. 209311; and variations thereof), pQE70, pQE60 and pQE-9, available from QIAGEN, Inc., *supra*; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman

(*Cell* 23:175 (1981)), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Introduction of the vector construct into the host cell can be effected by techniques known in the art which include, but are not limited to, calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, *Basic Methods In Molecular Biology* (1986).

In addition to encompassing host cells containing the vector constructs discussed herein, the invention also encompasses primary, secondary, and immortalized host cells of vertebrate origin, particularly mammalian origin, that have been engineered to delete or replace endogenous genetic material (e.g., TNFR coding sequence), and/or to include genetic material (e.g., heterologous polynucleotide sequences) that is operably associated with TNFR polynucleotides of the invention, and which activates, alters, and/or amplifies endogenous TNFR polynucleotides. For example, techniques known in the art may be used to operably associate heterologous control regions (e.g., promoter and/or enhancer) and endogenous TNFR polynucleotide sequences via homologous recombination (see, e.g., U.S. Patent No. 5,641,670, issued June 24, 1997; International application publication number WO 96/29411, published September 26, 1996; International application publication number WO 94/12650, published August 4, 1994; Koller *et al.*, *Proc. Natl. Acad. Sci.*

USA 86:8932-8935 (1989); and Zijlstra *et al.*, *Nature* 342:435-438 (1989), the disclosures of each of which are incorporated by reference in their entireties).

The host cells described *infra* can be used in a conventional manner to produce the gene product encoded by the recombinant sequence.

5 Alternatively, cell-free translation systems can also be employed to produce the polypeptides of the invention using RNAs derived from the DNA constructs of the present invention.

10 The polypeptide of the invention may be expressed or synthesized in a modified form, such as a fusion protein (comprising the polypeptide joined via a peptide bond to a heterologous protein sequence (of a different protein), e.g., the signal peptide of CK-beta8 (amino acids -21 to -1 of the CK-\_8 sequence disclosed in published PCT application PCT/US95/09058; filed 6/23/95) or the signal peptide of stanniocalcin (See ATCC Accession No. 75652, deposited January 25, 1994)), and may include not only secretion signals, but also additional heterologous functional regions. Such a fusion protein can be made by ligating polynucleotides of the invention and the desired nucleic acid sequence encoding the desired amino acid sequence to each other, by methods known in the art, in the proper reading frame, and expressing the fusion protein product by methods known in the art. Alternatively, such a fusion protein can be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art.

A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to stabilize and purify proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5 has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *J. Molecular Recognition* 8:52-58 (1995) and K. Johanson *et al.*, *J. Biol. Chem.* 270:9459-9471 (1995). In another example, preferred fusion proteins of the invention comprise a portion of an immunoglobulin light chain (i.e., a portion of a kappa or lambda light chain). In specific embodiments the fusion proteins of the invention comprise a portion of the constant region of a kappa or lambda light chain.

Proteins of the present invention include: products purified from natural sources, including bodily fluids, tissues and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also

include an initial modified methionine residue, in some cases as a result of host-mediated processes.

Proteins of the invention can be chemically synthesized using techniques known in the art (e.g., see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., N.Y., and Hunkapiller, M., et al., *Nature* 310:105-111 (1984)). For example, a peptide corresponding to a fragment of the complete TNFR (i.e., TNFR-6 $\alpha$  and/or TNFR-6 $\beta$ ) polypeptides of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the TNFR polypeptide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, a-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, g-Abu, e-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, b-alanine, fluoro-amino acids, designer amino acids such as b-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

The TNFR-6 alpha and/or TNFR-6 beta proteins may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given TNFR-6 alpha and/or TNFR-6 beta protein. Also, a given TNFR-6 alpha and/or TNFR-6 beta protein may contain many types of modifications. TNFR-6 alpha and/or TNFR-6 beta proteins may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic TNFR-6

alpha and/or TNFR-6 beta proteins may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); POST-TRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter *et al.*, *Meth Enzymol* 182:626-646 (1990); Rattan *et al.*, *Ann NY Acad Sci* 663:48-62 (1992).)

The invention encompasses TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  proteins which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to, specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH<sub>4</sub>, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin; etc.

Additional post-translational modifications encompassed by the invention include, for example, e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of prokaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Also provided by the invention are chemically modified derivatives of TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see US Patent Number 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog). For example, the polyethylene glycol may have an average molecular weight of about 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500,

7000, 7500, 8000, 8500, 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000,  
12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500,  
17,000, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 25,000, 30,000,  
35,000, 40,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, 80,000,  
85,000, 90,000, 95,000, or 100,000 kDa.

As noted above, the polyethylene glycol may have a branched structure. Branched polyethylene glycols are described, for example, in U.S. Patent No. 5,643,575; Morpurgo *et al.*, *Appl. Biochem. Biotechnol.* 56:59-72 (1996); Vorobjev *et al.*, *Nucleosides Nucleotides* 18:2745-2750 (1999); and Caliceti *et al.*, *Bioconjug. Chem.* 10:638-646 (1999), the disclosures of each of which are incorporated herein by reference.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g., EP 0 401 384, herein incorporated by reference (coupling PEG to G-CSF), see also Malik *et al.*, *Exp. Hematol.* 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulphydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

As suggested above, polyethylene glycol may be attached to proteins via linkage to any of a number of amino acid residues. For example,

polyethylene glycol can be linked to a proteins via covalent bonds to lysine, histidine, aspartic acid, glutamic acid, or cysteine residues. One or more reaction chemistries may be employed to attach polyethylene glycol to specific amino acid residues (e.g., lysine, histidine, aspartic acid, glutamic acid, or cysteine) of the protein or to more than one type of amino acid residue (e.g., lysine, histidine, aspartic acid, glutamic acid, cysteine and combinations thereof) of the protein.

One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective proteins chemically modified at the N-terminus modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

As indicated above, pegylation of the proteins of the invention may be accomplished by any number of means. For example, polyethylene glycol may be attached to the protein either directly or by an intervening linker. Linkerless systems for attaching polyethylene glycol to proteins are described in Delgado *et al.*, *Crit. Rev. Thera. Drug Carrier Sys.* 9:249-304 (1992); Francis *et al.*, *Intern. J. of Hematol.* 68:1-18 (1998); U.S. Patent No.

4,002,531; U.S. Patent No. 5,349,052; WO 95/06058; and WO 98/32466, the disclosures of each of which are incorporated herein by reference.

One system for attaching polyethylene glycol directly to amino acid residues of proteins without an intervening linker employs tresylated MPEG, which is produced by the modification of monmethoxy polyethylene glycol (MPEG) using tresylchloride ( $\text{ClSO}_2\text{CH}_2\text{CF}_3$ ). Upon reaction of protein with tresylated MPEG, polyethylene glycol is directly attached to amine groups of the protein. Thus, the invention includes protein-polyethylene glycol conjugates produced by reacting proteins of the invention with a polyethylene glycol molecule having a 2,2,2-trifluoroethane sulphonyl group.

Polyethylene glycol can also be attached to proteins using a number of different intervening linkers. For example, U.S. Patent No. 5,612,460, the entire disclosure of which is incorporated herein by reference, discloses urethane linkers for connecting polyethylene glycol to proteins. Protein-polyethylene glycol conjugates wherein the polyethylene glycol is attached to the protein by a linker can also be produced by reaction of proteins with compounds such as MPEG-succinimidylsuccinate, MPEG activated with 1,1'-carbonyldiimidazole, MPEG-2,4,5-trichloropenylcarbonate, MPEG-p-nitrophenolcarbonate, and various MPEG-succinate derivatives. A number additional polyethylene glycol derivatives and reaction chemistries for attaching polyethylene glycol to proteins are described in WO 98/32466, the entire disclosure of which is incorporated herein by reference. Pegylated protein products produced using the reaction chemistries set out herein are included within the scope of the invention.

The number of polyethylene glycol moieties attached to each protein of the invention (*i.e.*, the degree of substitution) may also vary. For example, the pegylated proteins of the invention may be linked, on average, to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, or more polyethylene glycol molecules. Similarly, the average degree of substitution within ranges such as 1-3, 2-4, 3-

5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-11, 10-12, 11-13, 12-14, 13-15, 14-16, 15-17, 16-18, 17-19, or 18-20 polyethylene glycol moieties per protein molecule.

Methods for determining the degree of substitution are discussed, for example, in Delgado *et al.*, *Crit. Rev. Thera. Drug Carrier Sys.* 9:249-304 (1992).

5           The TNFR proteins can be recovered and purified by known methods which include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

### **TNFR Proteins**

15           The invention further provides for the proteins containing polypeptide sequences encoded by the polynucleotides of the invention.

20           The TNFR proteins of the invention may be in monomers or multimers (i.e., dimers, trimers, tetramers, and higher multimers). Accordingly, the present invention relates to monomers and multimers of the TNFR proteins of the invention, their preparation, and compositions (preferably, pharmaceutical compositions) containing them. In specific embodiments, the polypeptides of the invention are monomers, dimers, trimers or tetramers. In additional embodiments, the multimers of the invention are at least dimers, at least trimers, or at least tetramers.

25           Multimers encompassed by the invention may be homomers or heteromers. As used herein, the term homomer, refers to a multimer containing only TNFR proteins of the invention (including TNFR fragments, variants, and fusion proteins, as described herein). These homomers may contain

TNFR proteins having identical or different polypeptide sequences. In a specific embodiment, a homomer of the invention is a multimer containing only TNFR proteins having an identical polypeptide sequence. In another specific embodiment, a homomer of the invention is a multimer containing TNFR proteins having different polypeptide sequences. In specific embodiments, the multimer of the invention is a homodimer (e.g., containing TNFR proteins having identical or different polypeptide sequences) or a homotrimer (e.g., containing TNFR proteins having identical or different polypeptide sequences). In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

As used herein, the term heteromer refers to a multimer containing heterologous proteins (i.e., proteins containing only polypeptide sequences that do not correspond to a polypeptide sequences encoded by the TNFR gene) in addition to the TNFR proteins of the invention. In a specific embodiment, the multimer of the invention is a heterodimer, a heterotrimer, or a heterotetramer. In additional embodiments, the heteromeric multimer of the invention is at least a heterodimer, at least a heterotrimer, or at least a heterotetramer.

Multimers of the invention may be the result of hydrophobic, hydrophilic, ionic and/or covalent associations and/or may be indirectly linked, by for example, liposome formation. Thus, in one embodiment, multimers of the invention, such as, for example, homodimers or homotrimers, are formed when proteins of the invention contact one another in solution. In another embodiment, heteromultimers of the invention, such as, for example, heterotrimers or heterotetramers, are formed when proteins of the invention contact antibodies to the polypeptides of the invention (including antibodies to the heterologous polypeptide sequence in a fusion protein of the invention) in solution. In other embodiments, multimers of the invention are formed by

covalent associations with and/or between the TNFR proteins of the invention. Such covalent associations may involve one or more amino acid residues contained in the polypeptide sequence of the protein (e.g., the polypeptide sequence recited in SEQ ID NO:2 or SEQ ID NO:4, contained in the polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97810), contained in the polypeptide encoded by the cDNA clone contained in ATCC Deposit No. 97809). In one instance, the covalent associations are cross-linking between cysteine residues located within the polypeptide sequences of the proteins which interact in the native (i.e., naturally occurring) polypeptide. In another instance, the covalent associations are the consequence of chemical or recombinant manipulation. Alternatively, such covalent associations may involve one or more amino acid residues contained in the heterologous polypeptide sequence in a TNFR fusion protein. In one example, covalent associations are between the heterologous sequence contained in a fusion protein of the invention (see, e.g., US Patent Number 5,478,925). In a specific example, the covalent associations are between the heterologous sequence contained in a TNFR-Fc fusion protein of the invention (as described herein). In another specific example, covalent associations of fusion proteins of the invention are between heterologous polypeptide sequences from another TNF family ligand/receptor member that is capable of forming covalently associated multimers, such as for example, oseteoprotegerin (see, e.g., International application publication number WO 98/49305, the contents of which are herein incorporated by reference in its entirety). In another embodiment, two or more TR6-alpha and/or TR6-beta polypeptides of the invention are joined through peptide linkers. Examples include those peptide linkers described in U.S. Pat. No. 5,073,627 (hereby incorporated by reference). Proteins comprising multiple TR6-alpha and/or TR6-beta polypeptides separated by peptide linkers may be produced using conventional recombinant DNA technology.

Another method for preparing multimer TR6-alpha and/or TR6-beta polypeptides of the invention involves use of TR6-alpha and/or TR6-beta polypeptides fused to a leucine zipper or isoleucine zipper polypeptide sequence. Leucine zipper and isoleucine zipper domains are polypeptides that promote multimerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, (1988)), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize.

Examples of leucine zipper domains suitable for producing soluble multimeric TR6-alpha and/or TR6-beta proteins are those described in PCT application WO 94/10308, hereby incorporated by reference. Recombinant fusion proteins comprising a soluble TR6-alpha and/or TR6-beta polypeptide fused to a peptide that dimerizes or trimerizes in solution are expressed in suitable host cells, and the resulting soluble multimeric TR6-alpha and/or TR6-beta is recovered from the culture supernatant using techniques known in the art.

Certain members of the TNF family of proteins are believed to exist in trimeric form (Beutler and Huffel, *Science* 264:667, 1994; Banner et al., *Cell* 73:431, 1993). Thus, trimeric TR6-alpha and/or TR6-beta may offer the advantage of enhanced biological activity. Preferred leucine zipper moieties are those that preferentially form trimers. One example is a leucine zipper derived from lung surfactant protein D (SPD), as described in Hoppe et al. (*FEBS Letters* 344:191, (1994)) and in U.S. patent application Ser. No. 08/446,922, hereby incorporated by reference. Other peptides derived from naturally occurring trimeric proteins may be employed in preparing trimeric TR6-alpha and/or TR6-beta.

In further preferred embodiments, TR6-alpha or TR6-beta polynucleotides of the invention are fused to a polynucleotide encoding a "FLAG" polypeptide. Thus, a TR6-alpha-FLAG or a TR6-beta-FLAG

fusion protein is encompassed by the present invention. The FLAG antigenic polypeptide may be fused to a TR6-alpha or a TR6-beta polypeptide of the invention at either or both the amino or the carboxy terminus. In preferred embodiments, a TR6-alpha-FLAG or a TR6-beta-FLAG fusion protein is expressed from a pFLAG-CMV-5a or a pFLAG-CMV-1 expression vector (available from Sigma, St. Louis, MO, USA). See, Andersson, S., et al., *J. Biol. Chem.* 264:8222-29 (1989); Thomsen, D. R., et al., *Proc. Natl. Acad. Sci. USA*, 81:659-63 (1984); and Kozak, M., *Nature* 308:241 (1984) (each of which is hereby incorporated by reference). In further preferred embodiments, a TR6-alpha-FLAG or a TR6-beta-FLAG fusion protein is detectable by anti-FLAG monoclonal antibodies (also available from Sigma).

The multimers of the invention may be generated using chemical techniques known in the art. For example, proteins desired to be contained in the multimers of the invention may be chemically cross-linked using linker molecules and linker molecule length optimization techniques known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, multimers of the invention may be generated using techniques known in the art to form one or more inter-molecule cross-links between the cysteine residues located within the polypeptide sequence of the proteins desired to be contained in the multimer (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Further, proteins of the invention may be routinely modified by the addition of cysteine or biotin to the C terminus or N-terminus of the polypeptide sequence of the protein and techniques known in the art may be applied to generate multimers containing one or more of these modified proteins (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, techniques known in the art may be applied to generate liposomes containing the protein components desired to be contained in the multimer of the invention (see, e.g., US Patent Number

5,478,925, which is herein incorporated by reference in its entirety).

Alternatively, multimers of the invention may be generated using genetic engineering techniques known in the art. In one embodiment, proteins contained in multimers of the invention are produced recombinantly using fusion protein technology described herein or otherwise known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In a specific embodiment, polynucleotides coding for a homodimer of the invention are generated by ligating a polynucleotide sequence encoding a polypeptide of the invention to a sequence encoding a linker polypeptide and then further to a synthetic polynucleotide encoding the translated product of the polypeptide in the reverse orientation from the original C-terminus to the N-terminus (lacking the leader sequence) (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In another embodiment, recombinant techniques described herein or otherwise known in the art are applied to generate recombinant polypeptides of the invention which contain a transmembrane domain and which can be incorporated by membrane reconstitution techniques into liposomes (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

In one embodiment, the invention provides isolated TNFR proteins comprising, or alternatively, consisting of, the amino acid sequence of the complete (full-length) TNFR polypeptide encoded by the cDNA contained in ATCC Deposit No. 97810, the amino acid sequence of the complete (full-length) TNFR polypeptide encoded by the cDNA contained in ATCC Deposit No. 97809, the amino acid sequence of the complete TNFR-6 $\alpha$  polypeptide disclosed in Figure 1 (SEQ ID NO:2), the amino acid sequence of the complete TNFR-6 $\beta$  polypeptide disclosed in <sup>Figures 2A - B</sup> ~~Figure 2~~ (SEQ ID NO:4), or a portion of the above polypeptides.

In another embodiment, the invention provides isolated TNFR proteins comprising, or alternatively consisting of, the amino acid sequence of the mature TNFR polypeptide encoded by the cDNA contained in ATCC Deposit No. 97810, the amino acid sequence of the mature TNFR polypeptide encoded by the cDNA contained in ATCC Deposit No. 97809, amino acid residues 31 to 300 of the TNFR-6 $\alpha$  sequence disclosed in Figure 1 (SEQ ID NO:2), amino acid residues 31 to 170 of the TNFR-6 $\beta$  sequence disclosed in <sup>Figures 2A - B</sup> ~~Figure 2~~ (SEQ ID NO:4), or a portion (i.e., fragment) of the above polypeptides.

Polypeptide fragments of the present invention include polypeptides comprising or alternatively, consisting of, an amino acid sequence contained in SEQ ID NO:2, an amino acid sequence contained in SEQ ID NO:4, an amino acid sequence encoded by the cDNA plasmid deposited as ATCC Deposit No. 97810, an amino acid sequence encoded by the cDNA plasmid deposited as ATCC Deposit No. 97809, or an amino acid sequence encoded by a nucleic acid which hybridizes (e.g., under stringent hybridization conditions) to the nucleotide sequence of the cDNA contained in ATCC Deposit No. 97810 and/or 97809, or shown in Figures 1 and/or <sup>2A - B</sup> ~~2~~ (SEQ ID NO:1 and SEQ ID NO:3, respectively) or the complementary strand thereto. Polynucleotides that hybridize to these polynucleotide fragments are also encompassed by the invention. Protein fragments may be "free-standing," or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments that comprise or alternatively, consist of from amino acid residues: 1 to 31, 32 to 50, 51 to 100, 101 to 150, 151 to 200, 201 to 250, and/or 251 to 300 of SEQ ID NO:2. Additional representative examples of polypeptide fragments of the invention include polypeptide fragments that comprise, or alternatively,

consist of from amino acids 1 to 31, 32 to 70, 70 to 100, 100 to 125, 126 to 150, and/or 151 to 170 of SEQ ID NO:4. Moreover, polypeptide fragments can be at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175 or 200 amino acids in length. Polynucleotides encoding these polypeptides are also encompassed by the invention.

In specific embodiments, polypeptide fragments of the invention comprise, or alternatively consist of, amino acid residues: 100 to 150, 150 to 200, 200 to 300, 210 to 300, 220 to 300, 230 to 300, 240 to 300, 250 to 300, 260 to 300, 270 to 300, 280 to 300, and/or 290 to 300 as depicted in Figure 1 (SEQ ID NO:2). Polynucleotides encoding these polypeptides are also encompassed by the invention.

TNFR comprises two domains having different structural and functional properties. The amino terminal domain spanning residues 30 to 196 of SEQ ID NO:2 shows homology to other members of the TNFR family, through conservation of four cysteine rich domains characteristic of TNFR families. Amino acid sequences contained in each of the four domains include amino acid residues 34 to 70, 73 to 113, 115 to 150, and 153 to 193, of SEQ ID NO:2, respectively. The carboxy terminal domain, spanning amino acid residues 197 to 300 of SEQ ID NO:2, has no significant homology to any known sequences. Unlike a number of other TNF receptor family members, TNFR appears to be exclusively a secreted protein and does not appear to be synthesized as a membrane associated form. While the amino terminal domain of TNFR appears to be required for biological activity of TNFR, the carboxy-terminal domain appears to be important for multimerization of TNFR.

In one embodiment, the polypeptides of the invention comprise, or alternatively consist of, amino acid residues 34 to 70, 73 to 113, 115 to 150, and 153 to 193, and/or 30-196 of SEQ ID NO:2. Polynucleotides encoding these polypeptides are also encompassed by the invention.

In another embodiment, the polypeptides of the invention comprise, or alternatively consist of, amino acid residues 197 to 240, 241 to 270, 271-300, and/or 197 to 300 of SEQ ID NO:2. Polynucleotides encoding these polypeptides are also encompassed by the invention. Since these polypeptide sequences are believed to be associated with multimerization of TNFR, proteins having one or more of these polypeptide sequences would be expected to form dimers, trimers and higher multimers, which may have advantageous properties, such as, increased binding affinity, greater stability, and longer circulating half life compared to monomeric forms. In a specific embodiment, the invention provides for fusion proteins comprising fusions of one or more of the above polypeptides to a heterologous sequence of a cell signaling molecule, such as a receptor, an extracellular domain thereof, and an active fragment, derivative, or analog of a receptor or an extracellular domain. In a preferred embodiment, heterologous sequences are selected from the family of TNFR-like receptors. Such sequences preferably include functional extracellular ligand binding domains and lack functional transmembrane and/or cytoplasmic domains. Such fusion proteins are useful for detecting molecules which interact with the fused heterologous sequences and thereby identifying potential new receptors and ligands. The fusion proteins are also useful for treatment of a variety of disorders, for example, those related to receptor binding. In one embodiment, fusion proteins of the invention comprising TNF/TNFR and TNF receptor/TNFR sequences are used to treat TNF and TNF receptor mediated disorders, such as, inflammation, autoimmune diseases, cancer, and disorders associated with excessive or alternatively, reduced apoptosis.

Additional embodiments TNFR polypeptide fragments comprising, or alternatively, consisting of, functional regions of polypeptides of the invention, such as the Garnier-Robson alpha-regions, beta-regions, turn-regions, and coil-regions, Chou-Fasman alpha-regions, beta-regions, and

coil-regions, Kyte-Doolittle hydrophilic regions, Eisenberg alpha- and beta-amphipathic regions, Karplus-Schulz flexible regions, Emini surface-forming regions and Jameson-Wolf regions of high antigenic index set out in Figure 4 (Table I) and Figure 5 (Table 2) and as described herein. In a preferred embodiment, the polypeptide fragments of the invention are antigenic. The data presented in columns VIII, IX, XIII, and XIV of Tables I and II can be used to routinely determine regions of TNFR which exhibit a high degree of potential for antigenicity. Regions of high antigenicity are determined from the data presented in columns VIII, IX, XIII, and/or XIV by choosing values which represent regions of the polypeptide which are likely to be exposed on the surface of the polypeptide in an environment in which antigen recognition may occur in the process of initiation of an immune response. Among highly preferred fragments of the invention are those that comprise regions of TNFR that combine several structural features, such as several (e.g., 1, 2, 3 or 4) of the features set out above. Polynucleotides encoding these polypeptides are also encompassed by the invention.

The present invention encompasses polypeptides comprising, or alternatively consisting of, an epitope of the polypeptide having an amino acid sequence of SEQ ID NOS:2 and 4, respectively, or an epitope of the polypeptide sequence encoded by a polynucleotide sequence contained in deposited clone ATCC Deposit Number 97810 and 97809, respectively, or encoded by a polynucleotide that hybridizes to the complement of the sequence of SEQ ID NOS:1 and 3, respectively, or contained in deposited clone ATCC Deposit Number 97810 and 97809, respectively, under stringent hybridization conditions or lower stringency hybridization conditions as defined *supra*. The present invention further encompasses polynucleotide sequences encoding an epitope of a polypeptide sequence of the invention (such as, for example, the sequence disclosed in SEQ ID NOS:1 and/or 3), polynucleotide sequences of the complementary strand of a polynucleotide sequence encoding an epitope of the

invention, and polynucleotide sequences which hybridize to the complementary strand under stringent hybridization conditions or lower stringency hybridization conditions defined *supra*.

The term "epitopes," as used herein, refers to portions of a polypeptide having antigenic or immunogenic activity in an animal, preferably a mammal, and most preferably in a human. In a preferred embodiment, the present invention encompasses a polypeptide comprising an epitope, as well as the polynucleotide encoding this polypeptide. An "immunogenic epitope," as used herein, is defined as a portion of a protein that elicits an antibody response in an animal, as determined by any method known in the art, for example, by the methods for generating antibodies described infra. (See, for example, Geysen et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1983)). The term "antigenic epitope," as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art, for example, by the immunoassays described herein. Immunospecific binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate TNFR-specific antibodies include: a polypeptide comprising, or alternatively consisting of, amino acid residues from about Ala-31 to about Thr-46, from about Phe-57 to about Thr-117, from about Cys-132 to about Thr-175, from about Gly-185 to about Thr-194, from about Val-205 to about Asp-217, from about Pro-239 to about Leu-264, and from about Ala-283 to about Pro-298 in SEQ ID NO:2; and from about Ala-31 to about Thr-46, from about Phe-57 to about Gln-80, from about Glu-86 to about His-106, from about Thr-108 to about Phe-119, from about His-129 to about Val-138, and from about Gly-142 to about Pro-166 in SEQ ID NO:4. These polypeptide fragments have been determined to bear antigenic epitopes of the TNFR-6 alpha and TNFR-6 beta polypeptides

respectively, by the analysis of the Jameson-Wolf antigenic index, as shown in Figures 4 and 5, above.

Fragments that function as epitopes may be produced by any conventional means. (See, e.g., Houghten, Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985), further described in U.S. Patent No. 4,631,211).

In the present invention, antigenic epitopes preferably contain a sequence of at least 4, at least 5, at least 6, at least 7, more preferably at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, and, most preferably, between about 15 to about 30 amino acids. Preferred polypeptides comprising immunogenic or antigenic epitopes are at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acid residues in length. Antigenic epitopes are useful, for example, to raise antibodies, including monoclonal antibodies, that specifically bind the epitope. Antigenic epitopes can be used as the target molecules in immunoassays. (See, for instance, Wilson et al., Cell 37:767-778 (1984); Sutcliffe et al., *Science* 219:660-666 (1983)).

Similarly, immunogenic epitopes can be used, for example, to induce antibodies according to methods well known in the art. (See, for instance, Sutcliffe et al., *supra*; Wilson et al., *supra*; Chow et al., Proc. Natl. Acad. Sci. USA 82:910-914; and Bittle et al., J. Gen. Virol. 66:2347-2354 (1985). The polypeptides comprising one or more immunogenic epitopes may be presented for eliciting an antibody response together with a carrier protein, such as an albumin, to an animal system (such as, for example, rabbit or mouse), or, if the polypeptide is of sufficient length (at least about 25 amino acids), the polypeptide may be presented without a carrier. However, immunogenic epitopes comprising as few as 8 to 10 amino acids have been shown to be sufficient to raise antibodies capable of binding to, at the very least, linear epitopes in a denatured polypeptide (e.g., in Western blotting).

Epitope-bearing polypeptides of the present invention may be used to induce antibodies according to methods well known in the art including, but not

limited to, *in vivo* immunization, *in vitro* immunization, and phage display methods. See, e.g., Sutcliffe et al., *supra*; Wilson et al., *supra*, and Bittle et al., J. Gen. Virol., 66:2347-2354 (1985). If *in vivo* immunization is used, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine residues may be coupled to a carrier using a linker such as maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carriers using a more general linking agent such as glutaraldehyde.

5 Animals such as, for example, rabbits, rats, and mice are immunized with either free or carrier-coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 micrograms of peptide or carrier protein and Freund's adjuvant or any other adjuvant known for stimulating an immune response. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody

10 that can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies

15 according to methods well known in the art.

20 As one of skill in the art will appreciate, and as discussed above, the polypeptides of the present invention comprising an immunogenic or antigenic epitope can be fused to other polypeptide sequences. For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides.

25 Such fusion proteins may facilitate purification and may increase half-life *in vivo*. This has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the

heavy or light chains of mammalian immunoglobulins. See, e.g., EP 394,827; Traunecker et al., *Nature*, 331:84-86 (1988). IgG Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion desulfide bonds have also been found to be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone. See, e.g., Fountoulakis et al., *J. Biochem.*, 270:3958-3964 (1995). Nucleic acids encoding the above epitopes can also be recombined with a gene of interest as an epitope tag (e.g., the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix-binding domain for the fusion protein. Extracts from cells infected with the recombinant vaccinia virus are loaded onto Ni<sup>2+</sup> nitriloacetic acid-agarose column and histidine-tagged proteins can be selectively eluted with imidazole-containing buffers.

The techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling") may be employed to modulate the activities of TR6-alpha and/or TR6-beta thereby effectively generating agonists and antagonists of TR6-alpha and/or TR6-beta. *See generally*, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458, and Patten, P. A., et al., *Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, S. *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, L. O., et al., *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo, M. M. and Blasco, R. *Biotechniques* 24(2):308-13 (1998) (each of these patents and publications are hereby incorporated by reference). In one embodiment, alteration of TR6-alpha and/or TR6-beta polynucleotides and corresponding polypeptides may be achieved by DNA

shuffling. DNA shuffling involves the assembly of two or more DNA segments into a desired TR6-alpha and/or TR6-beta molecule by homologous, or site-specific, recombination. In another embodiment, TR6-alpha and/or TR6-beta polynucleotides and corresponding polypeptides may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of TR6-alpha and/or TR6-beta may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules. In preferred embodiments, the heterologous molecules are TNF-alpha, TNF-beta, lymphotoxin-alpha, lymphotoxin-beta, FAS ligand, APRIL. In further preferred embodiments, the heterologous molecules are any member of the TNF family.

Additionally, the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling") may be employed to modulate the activities of TNFR thereby effectively generating agonists and antagonists of TNFR. *See generally*, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458, and Patten *et al.*, *Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, *Trends Biotechnol.* 16(2):76-82 (1998); Hansson *et al.*, *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo and Blasco, *Biotechniques* 24(2):308-13 (1998) (each of these patents and publications are hereby incorporated by reference). In one embodiment, alteration of TNFR polynucleotides and corresponding polypeptides may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments into a desired TNFR molecule by homologous, or site-specific, recombination. In another embodiment, TNFR polynucleotides and corresponding polypeptides may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of TNFR may

be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules. In preferred embodiments, the heterologous molecules are include, but are not limited to, TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, FasL, CD27L, CD30L, CD40L, 4-1BBL, DcR3, OX40L, TNF-gamma (International Publication No. WO 96/14328), TRAIL, AIM-II (International Publication No. WO 97/34911), APRIL (*J. Exp. Med.* 188(6):1185-1190), endokine-alpha (International Publication No. WO 98/07880), neutrokinne alpha (International Publication No. WO 98/18921), TR6 (International Publication No. WO 98/30694), OPG, OX40, and nerve growth factor (NGF), and soluble forms of Fas, CD30, CD27, CD40 and 4-IBB, TR2 (International Publication No. WO 96/34095), DR3 (International Publication No. WO 97/33904), DR4 (International Publication No. WO 98/32856), TR5 (International Publication No. WO 98/30693), TR7 (International Publication No. WO 98/41629), TRANK, TR9 (International Publication No. WO 98/56892), TR10 (International Publication No. WO 98/54202), 312C2 (International Publication No. WO 98/06842), and TR12, and soluble forms CD154, CD70, and CD153. In further preferred embodiments, the heterologous molecules are any member of the TNF family.

To improve or alter the characteristics of a TNFR polypeptide, protein engineering may be employed. Recombinant DNA technology known to those skilled in the art can be used to create novel mutant proteins or "muteins" including single or multiple amino acid substitutions, deletions, additions or fusion proteins. Such modified polypeptides can show, e.g., enhanced activity or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions. For instance, for many proteins, including the extracellular domain of a membrane

associated protein or the mature form(s) of a secreted protein, it is known in the art that one or more amino acids may be deleted from the N-terminus or C-terminus without substantial loss of biological function. For instance, Ron *et al.*, *J. Biol. Chem.*, 268:2984-2988 (1993) reported modified KGF proteins that had heparin binding activity even if 3, 8, or 27 amino-terminal amino acid residues were missing.

In the present case, since the proteins of the invention are members of the TNFR polypeptide family, deletions of N-terminal amino acids up to the Cysteine at position 49 of SEQ ID NOS:2 and 4 (TNFR-6 alpha and TNFR-6 beta) may retain some biological activity such as, for example regulation of cellular proliferation and apoptosis (e.g., of lymphoid cells), ability to bind Fas ligand (FasL), and ability to bind AIM-II. Polypeptides having further N-terminal deletions including the Cys-49 residue in SEQ ID NOS:2 and 4, would not be expected to retain such biological activities because it is known that these residues in a TNFR-related polypeptide are required for forming a disulfide bridge to provide structural stability which is needed for receptor/ligand binding and signal transduction. However, even if deletion of one or more amino acids from the N-terminus of a protein results in modification of loss of one or more biological functions of the protein, other functional activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature TNFR or extracellular domain of TNFR protein generally will be retained when less than the majority of the residues of the complete TNFR, mature TNFR, or extracellular domain of TNFR are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.

Accordingly, the present invention further provides polypeptides comprising, or alternatively consisting of, one or more residues deleted from

the amino terminus of the amino acid sequence of the TNFR shown in SEQ ID NOS:2 and 4, up to the cysteine residue at position number 49, and polynucleotides encoding such polypeptides. In particular, the present invention provides TNFR polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues m-300 of Figure 1 (SEQ ID NO:2) and/or residues n-170 of <sup>Figures 2A-B</sup> Figure 2 (SEQ ID NO:4), where m and n are integers in the range of 1-49 and where 49 is the position of the first cysteine residue from the N-terminus of the complete TNFR-6 $\alpha$  and TNFR-6 $\beta$  polypeptides (shown in SEQ ID NOS:2 and 4, respectively) believed to be required for activity of the TNFR-6 $\alpha$  and TNFR-6 $\beta$  proteins.

More in particular, the invention provides polynucleotides encoding polypeptides having (i.e., comprising) or alternatively consisting of, the amino acid sequence of a member selected from the group consisting of residues: 1-300, 2-300, 3-300, 4-300, 5-300, 6-300, 7-300, 8-300, 9-300, 10-300, 11-300, 12-300, 13-300, 14-300, 15-300, 16-300, 17-300, 18-300, 19-300, 20-300, 21-300, 22-300, 23-300, 24-300, 25-300, 26-300, 27-300, 28-300, 29-300, 30-300, 31-300, 32-300, 33-300, 34-300, 35-300, 36-300, 37-300, 38-300, 39-300, 40-300, 41-300, 42-300, 43-300, 44-300, 45-300, 46-300, 47-300, 48-300, and 49-300 of SEQ ID NO:2; and 1-170, 2-170, 3-170, 4-170, 5-170, 6-170, 7-170, 8-170, 9-170, 10-170, 11-170, 12-170, 13-170, 14-170, 15-170, 16-170, 17-170, 18-170, 19-170, 20-170, 21-170, 22-170, 23-170, 24-170, 25-170, 26-170, 27-170, 28-170, 29-170, 30-170, 31-170, 32-170, 33-170, 34-170, 35-170, 36-170, 37-170, 38-170, 39-170, 40-170, 41-170, 42-170, 43-170, 44-170, 45-170, 46-170, 47-170, 48-170, and 49-170 of SEQ ID NO:4.

Polypeptides encoded by these polynucleotide fragments are also encompassed by the invention.

In a specific embodiment, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino

acid sequence of a member selected from the group consisting of residues: Val-30 to His-300 of SEQ ID NO:2. Polypeptides encoded by these polynucleotide fragments are also encompassed by the invention.

In other specific embodiments, the invention provides  
5 polynucleotides encoding polypeptides comprising, or alternatively  
consisting of, the amino acid sequence of a member selected from the group  
consisting of residues: P-23 to H-300, and/or P-34 to H-300 of SEQ ID  
NO:2. Polypeptides encoded by these polynucleotides are also  
encompassed by the invention.

10 As mentioned above, even if deletion of one or more amino acids from  
the N-terminus of a protein results in modification of loss of one or more  
biological functions of the protein, other functional activities (e.g., biological  
activities) may still be retained. Thus, the ability of shortened TNFR  
muteins to induce and/or bind to antibodies which recognize the complete or  
15 mature forms of the polypeptides generally will be retained when less than  
the majority of the residues of the complete or mature polypeptide are  
removed from the N-terminus. Whether a particular polypeptide lacking  
N-terminal residues of a complete polypeptide retains such immunologic  
activities can readily be determined by routine methods described herein and  
20 otherwise known in the art. It is not unlikely that a TNFR mutein with a  
large number of deleted N-terminal amino acid residues may retain some  
biological or immunogenic activities. In fact, peptides composed of as few as  
six TNFR amino acid residues may often evoke an immune response.

Accordingly, the present invention further provides polypeptides  
25 having one or more residues deleted from the amino terminus of the TNFR-  
 $\alpha$  amino acid sequence shown in Figure 1 (i.e., SEQ ID NO:2), up to the  
arginine residue at position number 295 and polynucleotides encoding such  
polypeptides. In particular, the present invention provides polypeptides  
comprising or alternatively consisting of, the amino acid of residues n<sup>1</sup>-300 of

Figure 1 (SEQ ID NO:2), where  $n^1$  is an integer from 49 to 295, corresponding to the position of the amino acid residue in Figure 1 (SEQ ID NO:2).

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of a member selected from the group consisting of residues of C-49 to H-300; A-50 to H-300; Q-51 to H-300; C-52 to H-300; P-53 to H-300; P-54 to H-300; G-55 to H-300; T-56 to H-300; F-57 to H-300; V-58 to H-300; Q-59 to H-300; R-60 to H-300; P-61 to H-300; C-62 to H-300; R-63 to H-300; R-64 to H-300; D-65 to H-300; S-66 to H-300; P-67 to H-300; T-68 to H-300; T-69 to H-300; C-70 to H-300; G-71 to H-300; P-72 to H-300; C-73 to H-300; P-74 to H-300; P-75 to H-300; R-76 to H-300; H-77 to H-300; Y-78 to H-300; T-79 to H-300; Q-80 to H-300; F-81 to H-300; W-82 to H-300; N-83 to H-300; Y-84 to H-300; L-85 to H-300; E-86 to H-300; R-87 to H-300; C-88 to H-300; R-89 to H-300; Y-90 to H-300; C-91 to H-300; N-92 to H-300; V-93 to H-300; L-94 to H-300; C-95 to H-300; G-96 to H-300; E-97 to H-300; R-98 to H-300; E-99 to H-300; E-100 to H-300; E-101 to H-300; A-102 to H-300; R-103 to H-300; A-104 to H-300; C-105 to H-300; H-106 to H-300; A-107 to H-300; T-108 to H-300; H-109 to H-300; N-110 to H-300; R-111 to H-300; A-112 to H-300; C-113 to H-300; R-114 to H-300; C-115 to H-300; R-116 to H-300; T-117 to H-300; G-118 to H-300; F-119 to H-300; F-120 to H-300; A-121 to H-300; H-122 to H-300; A-123 to H-300; G-124 to H-300; F-125 to H-300; C-126 to H-300; L-127 to H-300; E-128 to H-300; H-129 to H-300; A-130 to H-300; S-131 to H-300; C-132 to H-300; P-133 to H-300; P-134 to H-300; G-135 to H-300; A-136 to H-300; G-137 to H-300; V-138 to H-300; I-139 to H-300; A-140 to H-300; P-141 to H-300; G-142 to H-300; T-143 to H-300; P-144 to H-300; S-145 to H-300; Q-146 to H-300; N-147 to H-300; T-148 to H-300; Q-149 to H-300; C-150 to H-300; Q-151 to H-300; P-152 to H-300;

C-153 to H-300; P-154 to H-300; P-155 to H-300; G-156 to H-300; T-157  
to H-300; F-158 to H-300; S-159 to H-300; A-160 to H-300; S-161 to  
H-300; S-162 to H-300; S-163 to H-300; S-164 to H-300; S-165 to H-300;  
E-166 to H-300; Q-167 to H-300; C-168 to H-300; Q-169 to H-300; P-170  
5 to H-300; H-171 to H-300; R-172 to H-300; N-173 to H-300; C-174 to  
H-300; T-175 to H-300; A-176 to H-300; L-177 to H-300; G-178 to H-300;  
L-179 to H-300; A-180 to H-300; L-181 to H-300; N-182 to H-300; V-183  
to H-300; P-184 to H-300; G-185 to H-300; S-186 to H-300; S-187 to  
H-300; S-188 to H-300; H-189 to H-300; D-190 to H-300; T-191 to H-300;  
10 L-192 to H-300; C-193 to H-300; T-194 to H-300; S-195 to H-300; C-196 to  
H-300; T-197 to H-300; G-198 to H-300; F-199 to H-300; P-200 to H-300;  
L-201 to H-300; S-202 to H-300; T-203 to H-300; R-204 to H-300; V-205 to  
H-300; P-206 to H-300; G-207 to H-300; A-208 to H-300; E-209 to H-300;  
E-210 to H-300; C-211 to H-300; E-212 to H-300; R-213 to H-300; A-214  
15 to H-300; V-215 to H-300; I-216 to H-300; D-217 to H-300; F-218 to  
H-300; V-219 to H-300; A-220 to H-300; F-221 to H-300; Q-222 to H-300;  
D-223 to H-300; I-224 to H-300; S-225 to H-300; I-226 to H-300; K-227 to  
H-300; R-228 to H-300; L-229 to H-300; Q-230 to H-300; R-231 to H-300;  
L-232 to H-300; L-233 to H-300; Q-234 to H-300; A-235 to H-300; L-236  
20 to H-300; E-237 to H-300; A-238 to H-300; P-239 to H-300; E-240 to  
H-300; G-241 to H-300; W-242 to H-300; G-243 to H-300; P-244 to H-300;  
T-245 to H-300; P-246 to H-300; R-247 to H-300; A-248 to H-300; G-249  
to H-300; R-250 to H-300; A-251 to H-300; A-252 to H-300; L-253 to  
H-300; Q-254 to H-300; L-255 to H-300; K-256 to H-300; L-257 to H-300;  
25 R-258 to H-300; R-259 to H-300; R-260 to H-300; L-261 to H-300; T-262  
to H-300; E-263 to H-300; L-264 to H-300; L-265 to H-300; G-266 to  
H-300; A-267 to H-300; Q-268 to H-300; D-269 to H-300; G-270 to H-300;  
A-271 to H-300; L-272 to H-300; L-273 to H-300; V-274 to H-300; R-275  
to H-300; L-276 to H-300; L-277 to H-300; Q-278 to H-300; A-279 to

H-300; L-280 to H-300; R-281 to H-300; V-282 to H-300; A-283 to H-300; R-284 to H-300; M-285 to H-300; P-286 to H-300; G-287 to H-300; L-288 to H-300; E-289 to H-300; R-290 to H-300; S-291 to H-300; V-292 to H-300; R-293 to H-300; E-294 to H-300; and R-295 to H-300 of the TNFR-  
5  $\alpha$  sequence shown in Figure 1 (SEQ ID NO:2). Polypeptides encoded by these polynucleotide fragments are also encompassed by the invention.

Similarly, many examples of biologically functional C-terminal deletion mutants are known. For instance, interferon gamma shows up to ten times higher activities by deleting 8-10 amino acid residues from the carboxy terminus of the protein (Döbeli *et al.*, *J. Biotechnology* 7:199-216 (1988)). In the present case, since the protein of the invention is a member of the TNFR polypeptide family, deletions of C-terminal amino acids up to the cysteine at position 193 and 132 of SEQ ID NOS:2 and 4, respectively, may retain some functional activity, such as, for example, a biological activity (such as, for example, regulation of proliferation and apoptosis (e.g., of lymphoid cells, ability to bind Fas ligand, and ability to bind AIM-II)). Polypeptides having further C-terminal deletions including the cysteines at positions 193 and 132 of SEQ ID NOS:2 and 4, respectively, would not be expected to retain such biological activities because it is known that these residues in TNF receptor-related polypeptides are required for forming disulfide bridges to provide structural stability which is needed for receptor binding.  
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However, even if deletion of one or more amino acids from the C-terminus of a protein results in modification or loss of one or more biological functions of the protein, other functional activities (e.g., biological activities, the ability to multimerize, and the ability to bind ligand (e.g., Fas ligand and AIM-II)) may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature form of the protein generally will be retained when less than the majority of the residues of the complete or mature form protein are removed from the  
25

C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.

Accordingly, the present invention further provides polypeptides having one or more residues from the carboxy terminus of the amino acid sequence of TNFR-6 alpha and TNFR-6 beta shown in SEQ ID NOS:2 and 4 up to the cysteine at position 193 and 132 of SEQ ID NOS:2 and 4, respectively, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of a member selected from the group consisting of residues 1-y and 1-z of the amino acid sequence in SEQ ID NOS:2 and 4, respectively, where y is any integer in the range of 193-300 and z is any integer in the range of 132-170. Polynucleotides encoding these polypeptides also are provided.

In certain preferred embodiments, the present invention provides polypeptides comprising, or alternatively, consisting of, the amino acid sequence of a member selected from the group consisting of residues 1-y' and 1-z' of the amino acid sequence in SEQ ID NOS:2 and 4, respectively, where y' is any integer in the range of 193-299 and z' is any integer in the range of 132-169. Polynucleotides encoding these polypeptides also are provided.

In additional preferred specific embodiments, the present invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of a member selected from the group consisting of residues Pro-23 to His-300, Val-30 to His-300, and Pro-34 to His-300 of SEQ ID NO:2 and polypeptides having the amino acid sequence of a member selected from the group consisting of residues Pro-23 to Pro-170, Val-30 to Pro-170, and Pro-34 to His-Pro-170 of SEQ ID NO:4. As described herein, these polypeptides may be fused to heterologous polypeptide sequences.

Polynucleotides encoding these polypeptides and these fusion polypeptides are also provided.

The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues m-y of SEQ ID NO:2 and n-z of SEQ ID NO:4, where m, n, y and z are integers as described above.

Also as mentioned above, even if deletion of one or more amino acids from the C-terminus of a protein results in modification or loss of one or more biological functions of the protein, other functional activities (e.g., biological activities, the ability to form homomultimers, and the ability to bind ligand (e.g., Fas ligand and AIM-II)) may still be retained. For example, the ability of the shortened TNFR mutein to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptide generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a TNFR mutein with a large number of deleted C-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six TNFR amino acid residues may often evoke an immune response.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the carboxy terminus of the amino acid sequence of the TNFR polypeptide shown in Figure 1 (SEQ ID NO:2), up to the glycine residue at position number 6, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues 1-m<sup>1</sup> of Figure 1 (i.e., SEQ ID NO:2), where m<sup>1</sup> is an

integer from 6 to 299, corresponding to the position of the amino acid residue in Figure 1 (SEQ ID NO:2).

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of a member selected from the group consisting of residues M-1 to V-299; M-1 to P-298; M-1 to L-297; M-1 to F-296; M-1 to R-295; M-1 to E-294; M-1 to R-293; M-1 to V-292; M-1 to S-291; M-1 to R-290; M-1 to E-289; M-1 to L-288; M-1 to G-287; M-1 to P-286; M-1 to M-285; M-1 to R-284; M-1 to A-283; M-1 to V-282; M-1 to R-281; M-1 to L-280; M-1 to A-279; M-1 to Q-278; M-1 to L-277; M-1 to L-276; M-1 to R-275; M-1 to V-274; M-1 to L-273; M-1 to L-272; M-1 to A-271; M-1 to G-270; M-1 to D-269; M-1 to Q-268; M-1 to A-267; M-1 to G-266; M-1 to L-265; M-1 to L-264; M-1 to E-263; M-1 to T-262; M-1 to L-261; M-1 to R-260; M-1 to R-259; M-1 to R-258; M-1 to L-257; M-1 to K-256; M-1 to L-255; M-1 to Q-254; M-1 to L-253; M-1 to A-252; M-1 to A-251; M-1 to R-250; M-1 to G-249; M-1 to A-248; M-1 to R-247; M-1 to P-246; M-1 to T-245; M-1 to P-244; M-1 to G-243; M-1 to W-242; M-1 to G-241; M-1 to E-240; M-1 to P-239; M-1 to A-238; M-1 to E-237; M-1 to L-236; M-1 to A-235; M-1 to Q-234; M-1 to L-233; M-1 to L-232; M-1 to R-231; M-1 to Q-230; M-1 to L-229; M-1 to R-228; M-1 to K-227; M-1 to I-226; M-1 to S-225; M-1 to I-224; M-1 to D-223; M-1 to Q-222; M-1 to F-221; M-1 to A-220; M-1 to V-219; M-1 to F-218; M-1 to D-217; M-1 to I-216; M-1 to V-215; M-1 to A-214; M-1 to R-213; M-1 to E-212; M-1 to C-211; M-1 to E-210; M-1 to E-209; M-1 to A-208; M-1 to G-207; M-1 to P-206; M-1 to V-205; M-1 to R-204; M-1 to T-203; M-1 to S-202; M-1 to L-201; M-1 to P-200; M-1 to F-199; M-1 to G-198; M-1 to T-197; M-1 to C-196; M-1 to S-195; M-1 to T-194; M-1 to C-193; M-1 to L-192; M-1 to T-191; M-1 to D-190; M-1 to H-189; M-1 to S-188; M-1 to S-187; M-1 to S-186; M-1 to G-185; M-1 to P-184; M-1 to V-183; M-1 to N-182; M-1 to L-181; M-1 to A-180; M-1 to

L-179; M-1 to G-178; M-1 to L-177; M-1 to A-176; M-1 to T-175; M-1 to C-174; M-1 to N-173; M-1 to R-172; M-1 to H-171; M-1 to P-170; M-1 to Q-169; M-1 to C-168; M-1 to Q-167; M-1 to E-166; M-1 to S-165; M-1 to S-164; M-1 to S-163; M-1 to S-162; M-1 to S-161; M-1 to A-160; M-1 to S-159; M-1 to F-158; M-1 to T-157; M-1 to G-156; M-1 to P-155; M-1 to P-154; M-1 to C-153; M-1 to P-152; M-1 to Q-151; M-1 to C-150; M-1 to Q-149; M-1 to T-148; M-1 to N-147; M-1 to Q-146; M-1 to S-145; M-1 to P-144; M-1 to T-143; M-1 to G-142; M-1 to P-141; M-1 to A-140; M-1 to I-139; M-1 to V-138; M-1 to G-137; M-1 to A-136; M-1 to G-135; M-1 to P-134; M-1 to P-133; M-1 to C-132; M-1 to S-131; M-1 to A-130; M-1 to H-129; M-1 to E-128; M-1 to L-127; M-1 to C-126; M-1 to F-125; M-1 to G-124; M-1 to A-123; M-1 to H-122; M-1 to A-121; M-1 to F-120; M-1 to F-119; M-1 to G-118; M-1 to T-117; M-1 to R-116; M-1 to C-115; M-1 to R-114; M-1 to C-113; M-1 to A-112; M-1 to R-111; M-1 to N-110; M-1 to H-109; M-1 to T-108; M-1 to A-107; M-1 to H-106; M-1 to C-105; M-1 to A-104; M-1 to R-103; M-1 to A-102; M-1 to E-101; M-1 to E-100; M-1 to E-99; M-1 to R-98; M-1 to E-97; M-1 to G-96; M-1 to C-95; M-1 to L-94; M-1 to V-93; M-1 to N-92; M-1 to C-91; M-1 to Y-90; M-1 to R-89; M-1 to C-88; M-1 to R-87; M-1 to E-86; M-1 to L-85; M-1 to Y-84; M-1 to N-83; M-1 to W-82; M-1 to F-81; M-1 to Q-80; M-1 to T-79; M-1 to Y-78; M-1 to H-77; M-1 to R-76; M-1 to P-75; M-1 to P-74; M-1 to C-73; M-1 to P-72; M-1 to G-71; M-1 to C-70; M-1 to T-69; M-1 to T-68; M-1 to P-67; M-1 to S-66; M-1 to D-65; M-1 to R-64; M-1 to R-63; M-1 to C-62; M-1 to P-61; M-1 to R-60; M-1 to Q-59; M-1 to V-58; M-1 to F-57; M-1 to T-56; M-1 to G-55; M-1 to P-54; M-1 to P-53; M-1 to C-52; M-1 to Q-51; M-1 to A-50; M-1 to C-49; M-1 to V-48; M-1 to L-47; M-1 to R-46; M-1 to E-45; M-1 to G-44; M-1 to T-43; M-1 to E-42; M-1 to A-41; M-1 to D-40; M-1 to R-39; M-1 to W-38; M-1 to P-37; M-1 to Y-36; M-1 to T-35; M-1 to P-34; M-1 to T-33; M-1 to E-32; M-1 to A-31; M-1 to V-30;

M-1 to G-29; M-1 to R-28; M-1 to V-27; M-1 to A-26; M-1 to P-25; M-1 to V-24; M-1 to P-23; M-1 to L-22; M-1 to L-21; M-1 to A-20; M-1 to P-19; M-1 to L-18; M-1 to A-17; M-1 to L-16; M-1 to V-15; M-1 to L-14; M-1 to C-13; M-1 to L-12; M-1 to L-11; M-1 to S-10; M-1 to L-9; M-1 to G-8; M-1 to P-7; and M-1 to G-6 of the sequence of the TNFR sequence shown in Figure 1 (SEQ ID NO:2). Polypeptides encoded by these polynucleotide fragments are also encompassed by the invention.

In specific embodiments, the invention provides polynucleotides encoding polypeptides comprising or alternatively consisting of the amino acid sequence of a member selected from the group consisting of residues: M-1 to A-271, M-1 to Q-254 and/or M-1 to F-221 of SEQ ID NO:2. Polypeptides encoded by these polynucleotide fragments are also encompassed by the invention.

The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini of a TNFR polypeptide, which may be described generally as having residues  $n^1-m^1$  of Figure 1 (i.e., SEQ ID NO:2), where  $n^1$  and  $m^1$  are integers as described above.

In additional embodiments, the present invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues  $30-m^3$  of Figure 1 (i.e., SEQ ID NO:2), where  $m^3$  is an integer from 36 to 299, corresponding to the position of the amino acid residue in Figure 1 (SEQ ID NO:2). For example, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of a member selected from the group consisting of residues V-30 to V-299; V-30 to P-298; V-30 to L-297; V-30 to F-296; V-30 to R-295; V-30 to E-294; V-30 to R-293; V-30 to V-292; V-30 to S-291; V-30 to R-290; V-30 to E-289; V-30 to L-288; V-30 to G-287; V-30 to P-286; V-30 to M-285; V-30 to R-284; V-30 to A-283; V-30 to V-282;

V-30 to R-281; V-30 to L-280; V-30 to A-279; V-30 to Q-278; V-30 to  
L-277; V-30 to L-276; V-30 to R-275; V-30 to V-274; V-30 to L-273; V-30  
to L-272; V-30 to A-271; V-30 to G-270; V-30 to D-269; V-30 to Q-268; V-  
30 to A-267; V-30 to G-266; V-30 to L-265; V-30 to L-264; V-30 to E-263;  
5 V-30 to T-262; V-30 to L-261; V-30 to R-260; V-30 to R-259; V-30 to  
R-258; V-30 to L-257; V-30 to K-256; V-30 to L-255; V-30 to Q-254; V-30  
to L-253; V-30 to A-252; V-30 to A-251; V-30 to R-250; V-30 to G-249; V-  
30 to A-248; V-30 to R-247; V-30 to P-246; V-30 to T-245; V-30 to P-244;  
10 V-30 to G-243; V-30 to W-242; V-30 to G-241; V-30 to E-240; V-30 to  
P-239; V-30 to A-238; V-30 to E-237; V-30 to L-236; V-30 to A-235; V-30  
to Q-234; V-30 to L-233; V-30 to L-232; V-30 to R-231; V-30 to Q-230; V-  
30 to L-229; V-30 to R-228; V-30 to K-227; V-30 to I-226; V-30 to S-225;  
15 V-30 to I-224; V-30 to D-223; V-30 to Q-222; V-30 to F-221; V-30 to  
A-220; V-30 to V-219; V-30 to F-218; V-30 to D-217; V-30 to I-216; V-30  
to V-215; V-30 to A-214; V-30 to R-213; V-30 to E-212; V-30 to C-211; V-  
30 to E-210; V-30 to E-209; V-30 to A-208; V-30 to G-207; V-30 to P-206;  
20 V-30 to V-205; V-30 to R-204; V-30 to T-203; V-30 to S-202; V-30 to  
L-201; V-30 to P-200; V-30 to F-199; V-30 to G-198; V-30 to T-197; V-30  
to C-196; V-30 to S-195; V-30 to T-194; V-30 to C-193; V-30 to L-192; V-  
30 to T-191; V-30 to D-190; V-30 to H-189; V-30 to S-188; V-30 to S-187;  
25 V-30 to S-186; V-30 to G-185; V-30 to P-184; V-30 to V-183; V-30 to  
N-182; V-30 to L-181; V-30 to A-180; V-30 to L-179; V-30 to G-178; V-30  
to L-177; V-30 to A-176; V-30 to T-175; V-30 to C-174; V-30 to N-173; V-  
30 to R-172; V-30 to H-171; V-30 to P-170; V-30 to Q-169; V-30 to C-168;  
V-30 to Q-167; V-30 to E-166; V-30 to S-165; V-30 to S-164; V-30 to S-163;  
V-30 to S-162; V-30 to S-161; V-30 to A-160; V-30 to S-159; V-30 to F-158;  
V-30 to T-157; V-30 to G-156; V-30 to P-155; V-30 to P-154; V-30 to  
C-153; V-30 to P-152; V-30 to Q-151; V-30 to C-150; V-30 to Q-149; V-30  
to T-148; V-30 to N-147; V-30 to Q-146; V-30 to S-145; V-30 to P-144; V-

30 to T-143; V-30 to G-142; V-30 to P-141; V-30 to A-140; V-30 to I-139; V-30 to V-138; V-30 to G-137; V-30 to A-136; V-30 to G-135; V-30 to P-134; V-30 to P-133; V-30 to C-132; V-30 to S-131; V-30 to A-130; V-30 to H-129; V-30 to E-128; V-30 to L-127; V-30 to C-126; V-30 to F-125; V-30 to G-124; V-30 to A-123; V-30 to H-122; V-30 to A-121; V-30 to F-120; V-30 to F-119; V-30 to G-118; V-30 to T-117; V-30 to R-116; V-30 to C-115; V-30 to R-114; V-30 to C-113; V-30 to A-112; V-30 to R-111; V-30 to N-110; V-30 to H-109; V-30 to T-108; V-30 to A-107; V-30 to H-106; V-30 to C-105; V-30 to A-104; V-30 to R-103; V-30 to A-102; V-30 to E-101; V-30 to E-100; V-30 to E-99; V-30 to R-98; V-30 to E-97; V-30 to G-96; V-30 to C-95; V-30 to L-94; V-30 to V-93; V-30 to N-92; V-30 to C-91; V-30 to Y-90; V-30 to R-89; V-30 to C-88; V-30 to R-87; V-30 to E-86; V-30 to L-85; V-30 to Y-84; V-30 to N-83; V-30 to W-82; V-30 to F-81; V-30 to Q-80; V-30 to T-79; V-30 to Y-78; V-30 to H-77; V-30 to R-76; V-30 to P-75; V-30 to P-74; V-30 to C-73; V-30 to P-72; V-30 to G-71; V-30 to C-70; V-30 to T-69; V-30 to T-68; V-30 to P-67; V-30 to S-66; V-30 to D-65; V-30 to R-64; V-30 to R-63; V-30 to C-62; V-30 to P-61; V-30 to R-60; V-30 to Q-59; V-30 to V-58; V-30 to F-57; V-30 to T-56; V-30 to G-55; V-30 to P-54; V-30 to P-53; V-30 to C-52; V-30 to Q-51; V-30 to A-50; V-30 to C-49; V-30 to V-48; V-30 to L-47; V-30 to R-46; V-30 to E-45; V-30 to G-44; V-30 to T-43; V-30 to E-42; V-30 to A-41; V-30 to D-40; V-30 to R-39; V-30 to W-38; V-30 to P-37; and V-30 to Y-36 of the sequence of the TFNR sequence shown in Figure 1 (SEQ ID NO:2).

Polypeptides encoded by these polynucleotide fragments are also encompassed by the invention. In specific embodiments, the invention provides polynucleotides encoding polypeptides comprising or alternatively consisting of the amino acid sequence of a member selected from the group consisting of residues: V-30 to A-271, V-30 to Q-254 and/or V-30 to F-221 of SEQ ID NO:2. Polypeptides encoded by these polynucleotides are also

encompassed by the invention. The present application is also directed to polynucleotides or polypeptides comprising, or alternatively, consisting of, a polynucleotide or polypeptide sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to a polypeptide or polypeptide sequence described above, respectively. The present invention also encompasses the above polynucleotide or polypeptide sequences fused to a heterologous polynucleotide or polypeptide sequence, respectively.

With respect to fragments of TNFR-6 $\beta$ , as mentioned above, even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the protein, other functional activities (e.g., biological activities, the ability to multimerize, the ability to bind ligand (e.g., Fas ligand and/or AIM-II)) may still be retained. For example, the ability of shortened TNFR muteins to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptides generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a TNFR mutein with a large number of deleted N-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six TNFR amino acid residues may often evoke an immune response.

Accordingly, the present invention further provides polypeptides comprising, or alternatively, consisting of, one or more residues deleted from the amino terminus of the TNFR-6 $\beta$  amino acid sequence shown in <sup>Figures 2A - B</sup> ~~Figure 2~~ (i.e., SEQ ID NO:4), up to the glycine residue at position number 165 and polynucleotides encoding such polypeptides. In particular, the present

invention provides polypeptides comprising, or alternatively consisting of,  
the amino acid sequence of residues n<sup>2</sup>-170 of <sup>Figures 2A-3</sup> Figure 2 (SEQ ID NO:4),  
where n<sup>2</sup> is an integer from 2 to 165, corresponding to the position of the  
amino acid residue in <sup>Figures 2A-3</sup> Figure 2 (SEQ ID NO:4).

More in particular, the invention provides polynucleotides encoding  
polypeptides comprising, or alternatively consisting of, the amino acid  
sequence of a member selected from the group consisting of residues of R-2  
to P-170; A-3 to P-170; L-4 to P-170; E-5 to P-170; G-6 to P-170; P-7 to  
P-170; G-8 to P-170; L-9 to P-170; S-10 to P-170; L-11 to P-170; L-12 to  
P-170; C-13 to P-170; L-14 to P-170; V-15 to P-170; L-16 to P-170; A-17 to  
P-170; L-18 to P-170; P-19 to P-170; A-20 to P-170; L-21 to P-170; L-22 to  
P-170; P-23 to P-170; V-24 to P-170; P-25 to P-170; A-26 to P-170; V-27 to  
P-170; R-28 to P-170; G-29 to P-170; V-30 to P-170; A-31 to P-170; E-32 to  
P-170; T-33 to P-170; P-34 to P-170; T-35 to P-170; Y-36 to P-170; P-37 to  
P-170; W-38 to P-170; R-39 to P-170; D-40 to P-170; A-41 to P-170; E-42  
to P-170; T-43 to P-170; G-44 to P-170; E-45 to P-170; R-46 to P-170; L-47  
to P-170; V-48 to P-170; C-49 to P-170; A-50 to P-170; Q-51 to P-170;  
C-52 to P-170; P-53 to P-170; P-54 to P-170; G-55 to P-170; T-56 to P-170;  
F-57 to P-170; V-58 to P-170; Q-59 to P-170; R-60 to P-170; P-61 to P-170;  
C-62 to P-170; R-63 to P-170; R-64 to P-170; D-65 to P-170; S-66 to P-170;  
P-67 to P-170; T-68 to P-170; T-69 to P-170; C-70 to P-170; G-71 to P-170;  
P-72 to P-170; C-73 to P-170; P-74 to P-170; P-75 to P-170; R-76 to P-170;  
H-77 to P-170; Y-78 to P-170; T-79 to P-170; Q-80 to P-170; F-81 to P-170;  
W-82 to P-170; N-83 to P-170; Y-84 to P-170; L-85 to P-170; E-86 to  
P-170; R-87 to P-170; C-88 to P-170; R-89 to P-170; Y-90 to P-170; C-91 to  
P-170; N-92 to P-170; V-93 to P-170; L-94 to P-170; C-95 to P-170; G-96 to  
P-170; E-97 to P-170; R-98 to P-170; E-99 to P-170; E-100 to P-170; E-101  
to P-170; A-102 to P-170; R-103 to P-170; A-104 to P-170; C-105 to P-170;  
H-106 to P-170; A-107 to P-170; T-108 to P-170; H-109 to P-170; N-110 to

P-170; R-111 to P-170; A-112 to P-170; C-113 to P-170; R-114 to P-170;  
C-115 to P-170; R-116 to P-170; T-117 to P-170; G-118 to P-170; F-119 to  
P-170; F-120 to P-170; A-121 to P-170; H-122 to P-170; A-123 to P-170;  
G-124 to P-170; F-125 to P-170; C-126 to P-170; L-127 to P-170; E-128 to  
P-170; H-129 to P-170; A-130 to P-170; S-131 to P-170; C-132 to P-170;  
P-133 to P-170; P-134 to P-170; G-135 to P-170; A-136 to P-170; G-137 to  
P-170; V-138 to P-170; I-139 to P-170; A-140 to P-170; P-141 to P-170;  
G-142 to P-170; E-143 to P-170; S-144 to P-170; W-145 to P-170; A-146 to  
P-170; R-147 to P-170; G-148 to P-170; G-149 to P-170; A-150 to P-170;  
P-151 to P-170; R-152 to P-170; S-153 to P-170; G-154 to P-170; G-155 to  
P-170; R-156 to P-170; R-157 to P-170; C-158 to P-170; G-159 to P-170;  
R-160 to P-170; G-161 to P-170; Q-162 to P-170; V-163 to P-170; A-164 to  
P-170; and G-165 to P-170 of the TNFR-6 $\beta$  sequence shown in <sup>Figures 2A-B</sup> Figure 2  
(SEQ ID NO:4). Polypeptides encoded by these polynucleotide fragments  
are also encompassed by the invention.

Also as mentioned above, even if deletion of one or more amino acids from the C-terminus of a protein results in modification of loss of one or more biological functions of the protein, other functional activities (e.g., biological activities, the ability to multimerize, ability to bind ligand (e.g., Fas ligand and/or AIM-II) may still be retained. For example, the ability of the shortened TNFR-6 $\beta$  mutein to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptide generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a TNFR-6 $\beta$  mutein with a large number of deleted C-terminal amino acid

residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six TNFR-6 $\beta$  amino acid residues may often evoke an immune response.

Accordingly, the present invention further provides polypeptides comprising, or alternatively consisting of one or more residues deleted from the carboxy terminus of the amino acid sequence of the TNFR-6 $\beta$  polypeptide shown in ~~Figure 2~~ (SEQ ID NO:4), up to the glycine residue at position number 6, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues 1-m<sup>2</sup> of ~~Figure 2A-B~~ (i.e., SEQ ID NO:2), where m<sup>2</sup> is an integer from 6 to 169, corresponding to the position of the amino acid residue in ~~Figure 2~~ (SEQ ID NO:4).

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of a member selected from the group consisting of residues M-1 to A-169; M-1 to L-168; M-1 to S-167; M-1 to P-166; M-1 to G-165; M-1 to A-164; M-1 to V-163; M-1 to Q-162; M-1 to G-161; M-1 to R-160; M-1 to G-159; M-1 to C-158; M-1 to R-157; M-1 to R-156; M-1 to G-155; M-1 to G-154; M-1 to S-153; M-1 to R-152; M-1 to P-151; M-1 to A-150; M-1 to G-149; M-1 to G-148; M-1 to R-147; M-1 to A-146; M-1 to W-145; M-1 to S-144; M-1 to E-143; M-1 to G-142; M-1 to P-141; M-1 to A-140; M-1 to I-139; M-1 to V-138; M-1 to G-137; M-1 to A-136; M-1 to G-135; M-1 to P-134; M-1 to P-133; M-1 to C-132; M-1 to S-131; M-1 to A-130; M-1 to H-129; M-1 to E-128; M-1 to L-127; M-1 to C-126; M-1 to F-125; M-1 to G-124; M-1 to A-123; M-1 to H-122; M-1 to A-121; M-1 to F-120; M-1 to F-119; M-1 to G-118; M-1 to T-117; M-1 to R-116; M-1 to C-115; M-1 to R-114; M-1 to C-113; M-1 to A-112; M-1 to R-111; M-1 to N-110; M-1 to H-109; M-1 to T-108; M-1 to A-107; M-1 to H-106; M-1 to C-105; M-1 to

A-104; M-1 to R-103; M-1 to A-102; M-1 to E-101; M-1 to E-100; M-1 to E-99; M-1 to R-98; M-1 to E-97; M-1 to G-96; M-1 to C-95; M-1 to L-94; M-1 to V-93; M-1 to N-92; M-1 to C-91; M-1 to Y-90; M-1 to R-89; M-1 to C-88; M-1 to R-87; M-1 to E-86; M-1 to L-85; M-1 to Y-84; M-1 to N-83; M-1 to W-82; M-1 to F-81; M-1 to Q-80; M-1 to T-79; M-1 to Y-78; M-1 to H-77; M-1 to R-76; M-1 to P-75; M-1 to P-74; M-1 to C-73; M-1 to P-72; M-1 to G-71; M-1 to C-70; M-1 to T-69; M-1 to T-68; M-1 to P-67; M-1 to S-66; M-1 to D-65; M-1 to R-64; M-1 to R-63; M-1 to C-62; M-1 to P-61; M-1 to R-60; M-1 to Q-59; M-1 to V-58; M-1 to F-57; M-1 to T-56; M-1 to G-55; M-1 to P-54; M-1 to P-53; M-1 to C-52; M-1 to Q-51; M-1 to A-50; M-1 to C-49; M-1 to V-48; M-1 to L-47; M-1 to R-46; M-1 to E-45; M-1 to G-44; M-1 to T-43; M-1 to E-42; M-1 to A-41; M-1 to D-40; M-1 to R-39; M-1 to W-38; M-1 to P-37; M-1 to Y-36; M-1 to T-35; M-1 to P-34; M-1 to T-33; M-1 to E-32; M-1 to A-31; M-1 to V-30; M-1 to G-29; M-1 to R-28; M-1 to V-27; M-1 to A-26; M-1 to P-25; M-1 to V-24; M-1 to P-23; M-1 to L-22; M-1 to L-21; M-1 to A-20; M-1 to P-19; M-1 to L-18; M-1 to A-17; M-1 to L-16; M-1 to V-15; M-1 to L-14; M-1 to C-13; M-1 to L-12; M-1 to L-11; M-1 to S-10; M-1 to L-9; M-1 to G-8; M-1 to P-7; and M-1 to G-6 of the sequence of the TNFR-6 $\beta$  shown in Figures 2A-B <sup>A</sup>Figure 2 (SEQ ID NO:4). Polypeptides encoded by these polynucleotide fragments are also encompassed by the invention.

The invention also provides polypeptides comprising, or alternatively consisting of, one or more amino acids deleted from both the amino and the carboxyl termini of a TNFR-6 $\beta$  polypeptide, which may be described generally as having residues  $n^2-m^2$  of Figures 2A-B <sup>A</sup>Figure 2 (i.e., SEQ ID NO:4), where  $n^2$  and  $m^2$  are integers as described above.

The present application is also directed to nucleic acid molecules comprising, or alternatively, consisting of, a polynucleotide sequence at least

90%, 92%, 95%, 96%, 97%, 98% or 99% identical to the polynucleotide sequence encoding a TNFR polypeptide set forth herein as m-y, n-z, n<sup>1</sup>-m<sup>1</sup>, 30-m<sup>3</sup>, and/or n<sup>2</sup>-m<sup>2</sup>. In preferred embodiments, the application is directed to nucleic acid molecules comprising, or alternatively, consisting of, a  
5 polynucleotide sequence at least 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to the polynucleotide sequences encoding polypeptides having the amino acid sequence of the specific N- and C-terminal deletions recited herein. The present invention also encompasses the above polynucleotide sequences fused to a heterologous polynucleotide sequence. Polypeptides encoded by these nucleic acids and/or polynucleotide sequences are also encompassed by the invention.  
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Also included are a nucleotide sequence encoding a polypeptide consisting of a portion of a complete TNFR amino acid sequence encoded by a cDNA clone contained in ATCC Deposit No. 97810, or 97809, where this portion excludes from 1 to about 49 amino acids from the amino terminus of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 and 97809, respectively, or from 1 to about 107 or 58 amino acids from the carboxy terminus of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 and 97809, respectively, or any combination of the above amino terminal and carboxy terminal deletions, of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809.  
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Polypeptides encoded by all of the above polynucleotides are also encompassed by the invention.

In addition to terminal deletion forms of the protein discussed above, it also will be recognized by one of ordinary skill in the art that some amino acid sequences of the TNFR polypeptides can be varied without significant effect on the structure or function of the proteins. If such differences in sequence are  
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contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

Thus, the invention further includes variations of the TNFR polypeptides which show substantial TNFR polypeptide functional activity (e.g., immunogenic activity, biological activity) or which include regions of TNFR protein such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions selected according to general rules known in the art so as have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie, J. U. *et al.*, *supra*, and the references cited therein.

Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe,

Tyr. Thus, the fragment, derivative or analog of the polypeptide of SEQ ID NO:2, 4 or 6, or that encoded by a deposited cDNA, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature or soluble extracellular polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids (such as, for example, an IgG Fc peptide fusion and/or an immunoglobulin light chain constant region peptide), a leader or secretory sequence, or a sequence which is employed for purification of the TNFR polypeptide) are fused to a TNFR polypeptide described herein. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Thus, the TNFR of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation. As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table III).

**TABLE III.** Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine

Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

5 Amino acids in the TNFR proteins of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for functional activity such as, for example, ligand/receptor (e.g., Fas ligand and/or AIM-II) receptor binding or *in vitro* or *in vitro* proliferative activity.

10 Of special interest are substitutions of charged amino acids with other charged or neutral amino acids which may produce proteins with highly desirable improved characteristics, such as less aggregation. Aggregation may not only reduce activity but also be problematic when preparing pharmaceutical formulations, because aggregates can be immunogenic (Pinckard *et al.*, *Clin. Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36: 838-845 (1987); Cleland *et al.*, *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993).

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Replacement of amino acids can also change the selectivity of the binding of a ligand to cell surface receptors. For example, Ostade *et al.*, *Nature*

361:266-268 (1993) describes certain mutations resulting in selective binding of TNF- $\alpha$  to only one of the two known types of TNF receptors. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al.* *Science* 255:306-312 (1992)).

Since TNFR-6 alpha and TNFR-6 beta are members of the TNF receptor-related protein family, to modulate rather than completely eliminate biological activities of TNFR preferably mutations are made in sequences encoding amino acids in the TNFR conserved extracellular domain, more preferably in residues within this region which are not conserved among members of the TNF receptor family. Also forming part of the present invention are isolated polynucleotides comprising nucleic acid sequences which encode the above TNFR mutants.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of the TNFR polypeptides can be substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988). Polypeptides of the invention also can be purified from natural or recombinant sources using anti-TNFR-6 alpha and TNFR-6 beta antibodies of the invention in methods which are well known in the art of protein purification.

The invention further provides isolated TNFR polypeptides comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of a full-length TNFR polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 or 4 or as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809; (b) the amino acid sequence of a mature TNFR polypeptide having the amino acid sequence at positions 31-300 in SEQ ID NO:2 or 31-170

in SEQ ID NO:4, or as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809; or (c) the amino acid sequence of a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809.

Further polypeptides of the present invention include polypeptides which have at least 90% similarity, more preferably at least 80%, 85%, 90%, 92%, or 95% similarity, and still more preferably at least 96%, 97%, 98% or 99% similarity to those described above. The polypeptides of the invention also comprise those which are at least 80% identical, more preferably at least 85%, 90%, 92% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide encoded by the deposited cDNA (ATCC Deposit Nos. 97810 or 97809) or to the polypeptide of SEQ ID NO:2 or 4, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By "% similarity" for two polypeptides is intended a similarity score produced by comparing the amino acid sequences of the two polypeptides using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) and the default settings for determining similarity. Bestfit uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2:482-489, 1981) to find the best segment of similarity between two sequences.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a TNFR polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to

five amino acid alterations per each 100 amino acids of the reference amino acid of the TNFR polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 80%, 85%, 90%, or 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 or 4, or to an amino acid sequence encoded by the cDNA contained in the deposits having ATCC Deposit No. 97810, or 97809, or fragments thereof (e.g., the sequence of any of the polypeptides corresponding to N or C terminal deletions of TNFR, as described herein (e.g., the polypeptide having the sequence of amino acids 30 to 300 of SEQ ID NO:2)) can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (*Comp. App. Biosci.* 6:237-245 (1990)). Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination of whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of this embodiment. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence. For example, a 90 amino acid residue subject sequence is aligned

with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C- termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

The polypeptide of the present invention have uses which include, but are not limited to, as molecular weight markers on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art. As described in detail below, the polypeptides of the present invention can also be used to raise polyclonal and monoclonal antibodies, which are useful in assays for detecting TNFR protein expression as described below or as agonists and antagonists capable of enhancing or inhibiting TNFR protein function. Further, such polypeptides can be used in the yeast two-hybrid system to "capture" TNFR protein binding proteins which are also candidate agonists and antagonists according to the present invention. The yeast two hybrid system is described in Fields and Song, *Nature* 340:245-246 (1989).

### ***Transgenics***

The proteins of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express polypeptides of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the transgene (i.e., polynucleotides of the invention) into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson *et al.*, *Appl. Microbiol. Biotechnol.* 40:691-698 (1994); Carver *et al.*, *Biotechnology (NY)* 11:1263-1270 (1993); Wright *et al.*, *Biotechnology (NY)* 9:830-834 (1991); and Hoppe *et al.*, U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, *Proc. Natl. Acad. Sci., USA* 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson *et al.*, *Cell* 56:313-321 (1989)); electroporation of cells or embryos (Lo, *Mol Cell. Biol.* 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer *et al.*, *Science* 259:1745 (1993); introducing nucleic acid constructs into embryonic pluripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano *et al.*, *Cell* 57:717-723 (1989)); etc. For a review of such techniques, see Gordon, "Transgenic Animals," *Intl. Rev. Cytol.* 115:171-229 (1989), which is incorporated by reference herein in its entirety. See also, U.S. Patent No. 5,464,764 (Capecchi, et al., Positive-Negative Selection Methods and Vectors); U.S. Patent No. 5,631,153 (Capecchi, et al., Cells and Non-

Human Organisms Containing Predetermined Genomic Modifications and Positive-Negative Selection Methods and Vectors for Making Same); U.S. Patent No. 4,736,866 (Leder, et al., Transgenic Non-Human Animals); and U.S. Patent No. 4,873,191 (Wagner, et al., Genetic Transformation of Zygotes); each of which is hereby incorporated by reference in its entirety. Further, the contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety.

Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell *et al.*, *Nature* 380:64-66 (1996); Wilmut *et al.*, *Nature* 385:810-813 (1997)), each of which is herein incorporated by reference in its entirety).

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals or chimeric animals. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (Lasko *et al.*, *Proc. Natl. Acad. Sci. USA* 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene.

The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu *et al.* (*Science* 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. The contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.

Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene on a

distinct background that is appropriate for an experimental model of interest.

Transgenic and "knock-out" animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of TNFR polypeptides, studying conditions and/or disorders associated with aberrant TNFR expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

In further embodiments of the invention, cells that are genetically engineered to express the proteins of the invention, or alternatively, that are genetically engineered not to express the proteins of the invention (e.g., knockouts) are administered to a patient *in vivo*. Such cells may be obtained from the patient (i.e., animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells, etc. The cells are genetically engineered *in vitro* using recombinant DNA techniques to introduce the coding sequence of polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, e.g., by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the polypeptides of the invention. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally. Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a

lymphatic or vascular graft. (See, for example, Anderson *et al.* US Patent No. 5,399,349; and Mulligan & Wilson, US Patent No. 5,460,959, each of which is incorporated by reference herein in its entirety).

When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

### ***Antibodies***

The present invention further relates to antibodies and T-cell antigen receptors (TCR) which immunospecifically bind a polypeptide, preferably an epitope, of the present invention (as determined by immunoassays well known in the art for assaying specific antibody-antigen binding). Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

Most preferably the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals.

Preferably, the antibodies are human, murine, donkey, sheep rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express endogenous immunoglobulins, as described infra and, for example in, U.S. Patent No. 5,939,598 by Kucherlapati et al.

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, et al., J. Immunol. 147:60-69 (1991); U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny et al., J. Immunol. 148:1547-1553 (1992).

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention that they recognize or specifically bind. The epitope(s) or polypeptide portion(s) may be

specified as described herein, e.g., by N-terminal and C-terminal positions, by size in contiguous amino acid residues, or listed in the Tables and Figures. Antibodies that specifically bind any epitope or polypeptide of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind polypeptides of the present invention, and allows for the exclusion of the same.

Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homolog of a polypeptide of the present invention are included.

Antibodies that bind polypeptides with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. Further included in the present invention are antibodies that bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or Kd less than  $5 \times 10^{-2} M$ ,  $10^{-2} M$ ,  $5 \times 10^{-3} M$ ,  $10^{-3} M$ ,  $5 \times 10^{-4} M$ ,  $10^{-4} M$ ,  $5 \times 10^{-5} M$ ,  $10^{-5} M$ ,  $5 \times 10^{-6} M$ ,  $10^{-6} M$ ,  $5 \times 10^{-7} M$ ,  $10^{-7} M$ ,  $5 \times 10^{-8} M$ ,  $10^{-8} M$ ,  $5 \times 10^{-9} M$ ,  $10^{-9} M$ ,  $5 \times 10^{-10} M$ ,  $10^{-10} M$ ,  $5 \times 10^{-11} M$ ,  $10^{-11} M$ ,  $5 \times 10^{-12} M$ ,  $10^{-12} M$ ,  $5 \times 10^{-13} M$ ,  $10^{-13} M$ ,  $5 \times 10^{-14} M$ ,  $10^{-14} M$ ,  $5 \times 10^{-15} M$ , and  $10^{-15} M$ .

The invention also provides antibodies that competitively inhibit binding of an antibody to an epitope of the invention as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody 5 competitively inhibits binding to the epitope by at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.

Antibodies of the present invention may act as agonists or antagonists of the polypeptides of the present invention. For example, the present invention includes antibodies which disrupt the receptor/ligand interactions with the 10 polypeptides of the invention either partially or fully. The invention features both receptor-specific antibodies and ligand-specific antibodies. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For 15 example, receptor activation can be determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or its substrate by immunoprecipitation followed by western blot analysis (for example, as described supra). In specific embodiments, antibodies are provided that inhibit ligand or receptor activity by at least 90%, at least 80%, at least 70%, at least 60%, or at 20 least 50% of the activity in absence of the antibody.

The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the receptor-ligand complex, and, preferably, do not specifically recognize the unbound receptor or the unbound ligand. Likewise, included in the invention are neutralizing antibodies which bind the ligand and prevent binding of the ligand to 25 the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included in the invention are antibodies which activate the receptor. These antibodies may act as receptor agonists, i.e., potentiate or activate either all

or a subset of the biological activities of the ligand-mediated receptor activation. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the peptides of the invention disclosed herein. The above antibody agonists can be made using methods known in the art. See, e.g., PCT publication WO 96/40281; U.S. Patent No. 5,811,097; Deng et al., Blood 92(6):1981-1988 (1998); Chen, et al., Cancer Res. 58(16):3668-3678 (1998); Harrop et al., J. Immunol. 161(4):1786-1794 (1998); Zhu et al., Cancer Res. 58(15):3209-3214 (1998); Yoon, et al., J. Immunol. 160(7):3170-3179 (1998); Prat et al., J. Cell. Sci. 111(Pt2):237-247 (1998); Pitard et al., J. Immunol. Methods 205(2):177-190 (1997); Liautard et al., Cytokine 9(4):233-241 (1997); Carlson et al., J. Biol. Chem. 272(17):11295-11301 (1997); Taryman et al., Neuron 14(4):755-762 (1995); Muller et al., Structure 6(9):1153-1167 (1998); Bartunek et al., Cytokine 8(1):14-20 (1996) (which are all incorporated by reference herein in their entireties).

Antibodies of the present invention may be used, for example, but not limited to, to purify, detect, and target the polypeptides of the present invention, including both in vitro and in vivo diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in biological samples. See, e.g., Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) (incorporated by reference herein in its entirety).

As discussed in more detail below, the antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as

heterologous polypeptides, drugs, or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387.

The antibodies of the invention include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

The antibodies of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to an antigen-of-interest can be produced by various procedures well known in the art. For example, a polypeptide of the invention can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lyssolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display

technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well-known in the art and are discussed in detail in Example 11. Briefly, mice can be immunized with a polypeptide of the invention or a cell expressing such peptide. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well-known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with an antigen of the invention with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')2 fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

For example, the antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular, such phage can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., J. Immunol. Methods 182:41-50 (1995); Ames et al., J. Immunol. Methods 184:177-186 (1995); Kettleborough et al., Eur. J. Immunol. 24:952-958 (1994); Persic et al., Gene 187 9-18 (1997); Burton et al., Advances in Immunology 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., BioTechniques 12(6):864-869 (1992); and Sawai et al., AJRI 34:26-34 (1995); and Better et al., Science 240:1041-1043 (1988) (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al., Methods in Enzymology 203:46-88 (1991); Shu et al., PNAS 90:7995-7999 (1993); and Skerra et al., Science 240:1038-1040 (1988). For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Gillies et al., (1989) J. Immunol. Methods 125:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816397, which are incorporated herein by reference in their entireties. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These

framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Patent No. 5,585,089; Riechmann et al., *Nature* 332:323 (1988), which are incorporated herein by reference in their entireties.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology* 28(4/5):489-498 (1991); Studnicka et al., *Protein Engineering* 7(6):805-814 (1994); Roguska. et al., *PNAS* 91:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous

deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring that express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology.

5 The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, Int. Rev. Immunol. 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 10 96/34096; WO 96/33735; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 15 5,569,825; 5,661,016; 5,545,806; 5,814,318; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

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25 Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al., Bio/technology 12:899-903 (1988)).

Further, antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" polypeptides of the invention using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona,

FASEB J. 7(5):437-444; (1989) and Nissinoff, J. Immunol. 147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide of the invention to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligands/receptors, and thereby block TNFR mediated inhibition of apoptosis.

#### Polynucleotides Encoding Antibodies.

The invention further provides polynucleotides comprising a nucleotide sequence encoding an antibody of the invention and fragments thereof. The invention also encompasses polynucleotides that hybridize under stringent or lower stringency hybridization conditions, e.g., as defined *supra*, to polynucleotides that encode an antibody, preferably, that specifically binds to a polypeptide of the invention, preferably, an antibody that binds to a polypeptide having the amino acid sequence of SEQ ID NO:2 or 4.

The polynucleotides may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., BioTechniques 17:242 (1994)), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid

encoding a particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the immunoglobulin may be obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the antibody, such as hybridoma cells selected to express an antibody of the invention) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

Once the nucleotide sequence and corresponding amino acid sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel et al., eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties ), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

In a specific embodiment, the amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the sequences of the complementarity determining regions (CDRs) by methods that are well known in the art, e.g., by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, e.g., into human framework regions to

humanize a non-human antibody, as described supra. The framework regions may be naturally occurring or consensus framework regions, and preferably human framework regions (see, e.g., Chothia et al., J. Mol. Biol. 278: 457-479 (1998) for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds a polypeptide of the invention. Preferably, as discussed supra, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. 81:851-855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described supra, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region, e.g., humanized antibodies.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,694,778; Bird, 1988, Science 242:423- 42; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-54) can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra et al., 1988, Science 242:1038- 1041).

#### Methods of Producing Antibodies

The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

Recombinant expression of an antibody of the invention, or fragment, derivative or analog thereof, e.g., a heavy or light chain of an antibody of the invention, requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the antibody molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody molecule of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant

antibody molecule, are used for the expression of a recombinant antibody molecule. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., 1986, Gene 45:101; Cockett et al., 1990, Bio/Technology 8:2).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the

virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non- essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (e.g., see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:51-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing

of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 3T3, WI38, and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT20 and T47D, and normal mammary gland cell line such as, for example, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibody molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 192, Proc. Natl. Acad. Sci. USA 48:202), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt-cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:357; O'Hare et al., 1981, Proc. Natl.

Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIB TECH 11(5):155-215); and hygro, which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, Current Protocols in Human Genetics, John Wiley & Sons, NY.; Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1, which are incorporated by reference herein in their entireties.

The expression levels of an antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., 1983, Mol. Cell. Biol. 3:257).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light

chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, Nature 322:52; Kohler, 1980, Proc. Natl. Acad. Sci. USA 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once an antibody molecule of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

#### Antibody conjugates

The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, preferably at least 10, 20 or 50 amino acids of the polypeptide) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, preferably at least 10, 20 or 50 amino acids of the polypeptide) of the present invention. For example, antibodies may be used to target the polypeptides of the present invention to particular cell types, either in vitro or in vivo, by fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to the polypeptides of the present invention may also be used in in vitro immunoassays and purification methods using methods known in the art. See e.g., Harbor et al., supra, and PCT publication WO 93/21232; EP 439,095; Naramura et al., Immunol. Lett. 39:91-99 (1994); U.S. Patent 5,474,981; Gillies et al., PNAS 89:1428-1432 (1992); Fell et al., J. Immunol. 146:2446-2452(1991), which are incorporated by reference in their entireties.

The present invention further includes compositions comprising the polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the constant region, hinge region, CH1 domain, CH2 domain, and CH3 domain or any combination of whole domains or portions thereof. The polypeptides may also be fused or conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. See, e.g., U.S. Patent Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 91/06570; Ashkenazi et al., Proc. Natl. Acad. Sci. USA 88:10535-10539 (1991); Zheng et al., J. Immunol. 154:5590-5600 (1995); and Vil et al., Proc. Natl. Acad. Sci. USA 89:11337- 11341(1992) (said references incorporated by reference in their entireties).

As discussed, supra, the polypeptides of the present invention may be fused or conjugated to the above antibody portions to increase the in vivo half life of the polypeptides or for use in immunoassays using methods known in the art. Further, the polypeptides of the present invention may be fused or conjugated to the above antibody portions to facilitate purification. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP 394,827; Traunecker et al., Nature 331:84-86 (1988). The polypeptides of the present invention fused or conjugated to an antibody having disulfide-linked dimeric structures (due to the

IgG) may also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis et al., J. Biochem. 270:3958-3964 (1995)). In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP A 232,262). Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, D. Bennett et al., J. Molecular Recognition 8:52-58 (1995); K. Johanson et al., J. Biol. Chem. 270:9459-9471 (1995)).

Moreover, the antibodies or fragments thereof of the present invention can be fused to marker sequences, such as a peptide to facilitate their purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., Cell 37:767 (1984)) and the "flag" tag.

The present invention further encompasses antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical testing procedure to, e.g., determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various

enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. See, for example, U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention.

5 Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{111}\text{In}$  or  $^{99}\text{Tc}$ .

Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, 20 dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis- dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin,

mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor,  $\alpha$ -interferon,  $\beta$ -interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic

Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev. 62:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety.

An antibody, with or without a therapeutic moiety conjugated to it, administered alone or in combination with cytotoxic factor(s) and/or cytokine(s) can be used as a therapeutic.

10                  Assays For Antibody Binding

The antibodies of the invention may be assayed for immunospecific binding by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X- 100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (e.g., EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (e.g., 1-4 hours) at 4°

C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, e.g., western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (e.g., pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), blocking the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, blocking the membrane with a secondary antibody (which recognizes the primary antibody, e.g., an anti-human antibody) conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e.g., 32P or 125I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding western blot protocols see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a

detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody  
5 (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be  
10 knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

15 The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., <sup>3</sup>H or <sup>125</sup>I) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for  
20 a particular antigen and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the antigen is incubated with antibody of interest is conjugated to a labeled compound (e.g., <sup>3</sup>H or <sup>125</sup>I) in the presence of increasing amounts of an unlabeled second antibody.

#### 25 Therapeutic Uses

The present invention is further directed to antibody-based therapies which involve administering antibodies of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating one or more of the

described disorders. Therapeutic compounds of the invention include, but are not limited to, antibodies of the invention (including fragments, analogs and derivatives thereof as described herein) and nucleic acids encoding antibodies of the invention (including fragments, analogs and derivatives thereof as described herein). The antibodies of the invention can be used to treat or prevent diseases and disorders associated with aberrant expression and/or activity of a polypeptide of the invention, including, but not limited to, diseases and/or disorders such as autoimmune diseases and/or deficiencies, as discussed herein. The treatment and/or prevention of diseases and disorders associated with aberrant expression and/or activity of a polypeptide of the invention includes, but is not limited to, alleviating symptoms associated with those diseases and disorders. Antibodies of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

A summary of the ways in which the antibodies of the present invention may be used therapeutically includes binding polynucleotides or polypeptides of the present invention locally or systemically in the body or by direct cytotoxicity of the antibody, e.g. as mediated by complement (CDC) or by effector cells (ADCC). Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

The antibodies of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic growth factors (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

The antibodies of the invention may be administered alone or in combination with other types of treatments (e.g., radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents).

Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, human antibodies, fragments derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

It is preferred to use high affinity and/or potent in vivo inhibiting and/or neutralizing antibodies against polypeptides or polynucleotides of the present invention, fragments or regions thereof, for both immunoassays directed to and therapy of disorders related to polynucleotides or polypeptides, including fragments thereof, of the present invention. Such antibodies, fragments, or regions, will preferably have an affinity for polynucleotides or polypeptides, including fragments thereof. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  $5 \times 10^{-6}$  M,  $10^{-6}$  M,  $5 \times 10^{-7}$  M,  $10^{-7}$  M,  $5 \times 10^{-8}$  M,  $10^{-8}$  M,  $5 \times 10^{-9}$  M,  $10^{-9}$  M,  $5 \times 10^{-10}$  M,  $10^{-10}$  M,  $5 \times 10^{-11}$  M,  $10^{-11}$  M,  $5 \times 10^{-12}$  M,  $10^{-12}$  M,  $5 \times 10^{-13}$  M,  $10^{-13}$  M,  $5 \times 10^{-14}$  M,  $10^{-14}$  M,  $5 \times 10^{-15}$  M, and  $10^{-15}$  M.

### Gene Therapy

In a specific embodiment, nucleic acids comprising sequences encoding antibodies or functional derivatives thereof, are administered to treat, inhibit or prevent a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95;

Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the compound comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody nucleic acids (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438). In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids in vitro, then transplanted into the patient. These two approaches are known, respectively, as in vivo or ex vivo gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered in vivo, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by

constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, e.g., by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a specific embodiment, viral vectors that contains nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller et al., 1993, Meth. Enzymol. 217:581-599). These retroviral vectors have been to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More

detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the mdrl gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy.

Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, Human Gene Therapy 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, Science 252:431-434; Rosenfeld et al., 1992, Cell 68:143-155; Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234; PCT Publication WO94/12649; and Wang, et al., 1995, Gene Therapy 2:775-783. In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300; U.S. Patent No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under

selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, e.g., Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (e.g., hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as Tlymphocytes, Blymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained in vitro can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, Cell 71:973-985; Rheinwald, 1980, Meth. Cell Bio. 21A:229; and Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

#### Demonstration of Therapeutic or Prophylactic Activity

The compounds or pharmaceutical compositions of the invention are preferably tested in vitro, and then in vivo for the desired therapeutic or prophylactic activity, prior to use in humans. For example, in vitro assays to demonstrate the therapeutic or prophylactic utility of a compound or pharmaceutical composition include, the effect of a compound on a cell line or a patient tissue sample. The effect of the compound or composition on the cell line and/or tissue sample can be determined utilizing techniques known to those of skill in the art including, but not limited to, rosette formation assays and cell lysis assays. In accordance with the invention, in vitro assays which can be used to determine whether administration of a specific compound is indicated, include in vitro cell culture assays in which a patient tissue sample is grown in culture, and

exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

Therapeutic/Prophylactic Administration and Composition

The invention provides methods of treatment, inhibition and prophylaxis by administration to a subject of an effective amount of a compound or pharmaceutical composition of the invention, preferably an antibody of the invention. In a preferred aspect, the compound is substantially purified (e.g., substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid or an immunoglobulin are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a compound of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes.

The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents.

Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compounds or compositions of the invention into

the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (see Langer, 1990, *Science* 249:1527-1533; Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, *CRC Crit. Ref. Biomed. Eng.* 14:201; Buchwald et al., 1980, *Surgery* 88:507; Saudek et al., 1989, *N. Engl. J. Med.* 321:574). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., 1983, *Macromol. Sci. Rev. Macromol. Chem.* 23:61; see also Levy et al., 1985, *Science*

228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J.Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

5 Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox- like peptide which is known to enter the nucleus (see e.g., Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is

administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline.

Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

#### Diagnosis and Imaging

Labeled antibodies, and derivatives and analogs thereof, which specifically bind to a polypeptide of interest can be used for diagnostic purposes to detect, diagnose, or monitor diseases and/or disorders associated with the aberrant expression and/or activity of a polypeptide of the invention. The invention provides for the detection of aberrant expression of a polypeptide of interest, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of aberrant expression.

The invention provides a diagnostic assay for diagnosing a disorder, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of a particular disorder. With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a

means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Assaying TR6-alpha and/or TR6-beta polypeptide levels in a biological sample can occur using antibody-based techniques. Antibodies of the invention can be used to assay protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (e.g., see Jalkanen, M., et al., *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., et al., *J. Cell. Biol.* 105:3087-3096 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine ( $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{123}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{115m}\text{In}$ ,  $^{113m}\text{In}$ ,  $^{112}\text{In}$ ,  $^{111}\text{In}$ ), and technetium ( $^{99}\text{Tc}$ ,  $^{99m}\text{Tc}$ ), thallium ( $^{201}\text{Ti}$ ), gallium ( $^{68}\text{Ga}$ ,  $^{67}\text{Ga}$ ), palladium ( $^{103}\text{Pd}$ ), molybdenum ( $^{99}\text{Mo}$ ), xenon ( $^{133}\text{Xe}$ ), fluorine ( $^{18}\text{F}$ ),  $^{153}\text{Sm}$ ,  $^{177}\text{Lu}$ ,  $^{159}\text{Gd}$ ,  $^{149}\text{Pm}$ ,  $^{140}\text{La}$ ,  $^{175}\text{Yb}$ ,  $^{166}\text{Ho}$ ,  $^{90}\text{Y}$ ,  $^{47}\text{Sc}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{142}\text{Pr}$ ,  $^{105}\text{Rh}$ ,  $^{97}\text{Ru}$ ; luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Techniques known in the art may be applied to label antibodies of the invention. Such techniques include, but are not limited to, the use of bifunctional conjugating agents (see e.g., U.S. Patent Nos. 5,756,065; 5,714,631; 5,696,239; 5,652,361; 5,505,931; 5,489,425; 5,435,990; 5,428,139; 5,342,604; 5,274,119; 4,994,560; and 5,808,003; the contents of each of which are hereby incorporated by reference in its entirety).

One aspect of the invention is the detection and diagnosis of a disease or disorder associated with aberrant expression of a polypeptide of the interest in an animal, preferably a mammal and most preferably a human. In one embodiment,

diagnosis comprises: a) administering (for example, parenterally, subcutaneously, or intraperitoneally) to a subject an effective amount of a labeled molecule which specifically binds to the polypeptide of interest; b) waiting for a time interval following the administering for permitting the labeled molecule to preferentially concentrate at sites in the subject where the polypeptide is expressed (and for unbound labeled molecule to be cleared to background level); c) determining background level; and d) detecting the labeled molecule in the subject, such that detection of labeled molecule above the background level indicates that the subject has a particular disease or disorder associated with aberrant expression of the polypeptide of interest. Background level can be determined by various methods including, comparing the amount of labeled molecule detected to a standard value previously determined for a particular system.

It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of  $^{99m}\text{Tc}$ . The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. *In vivo* tumor imaging is described in S.W. Burchiel et al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments." (Chapter 13 in *Tumor Imaging: The Radiochemical Detection of Cancer*, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982).

Depending on several variables, including the type of label used and the mode of administration, the time interval following the administration for permitting the labeled molecule to preferentially concentrate at sites in the subject and for unbound labeled molecule to be cleared to background level is 6 to 48 hours or 6 to 24 hours or 6 to 12 hours. In another embodiment the time interval following administration is 5 to 20 days or 5 to 10 days.

In an embodiment, monitoring of the disease or disorder is carried out by repeating the method for diagnosing the disease or disease, for example, one month after initial diagnosis, six months after initial diagnosis, one year after initial diagnosis, etc.

5           Presence of the labeled molecule can be detected in the patient using methods known in the art for in vivo scanning. These methods depend upon the type of label used. Skilled artisans will be able to determine the appropriate method for detecting a particular label. Methods and devices that may be used in the diagnostic methods of the invention include, but are not limited to, computed tomography (CT), whole body scan such as position emission tomography (PET), magnetic resonance imaging (MRI), and sonography.

10           In a specific embodiment, the molecule is labeled with a radioisotope and is detected in the patient using a radiation responsive surgical instrument (Thurston et al., U.S. Patent No. 5,441,050). In another embodiment, the molecule is labeled with a fluorescent compound and is detected in the patient using a fluorescence responsive scanning instrument. In another embodiment, the molecule is labeled with a positron emitting metal and is detected in the patent using positron emission-tomography. In yet another embodiment, the molecule is labeled with a paramagnetic label and is detected in a patient using magnetic resonance imaging (MRI).

### Kits

25           The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises an antibody of the invention, preferably a purified antibody, in one or more containers. In a specific embodiment, the kits of the present invention contain a substantially isolated polypeptide comprising an epitope which is specifically immunoreactive with an antibody included in the kit. Preferably, the kits of the present invention further comprise a control antibody which does not react with the polypeptide of interest. In another specific

embodiment, the kits of the present invention contain a means for detecting the binding of an antibody to a polypeptide of interest (e.g., the antibody may be conjugated to a detectable substrate such as a fluorescent compound, an enzymatic substrate, a radioactive compound or a luminescent compound, or a second antibody which recognizes the first antibody may be conjugated to a detectable substrate).

In another specific embodiment of the present invention, the kit is a diagnostic kit for use in screening serum containing antibodies specific against proliferative and/or cancerous polynucleotides and polypeptides. Such a kit may include a control antibody that does not react with the polypeptide of interest. Such a kit may include a substantially isolated polypeptide antigen comprising an epitope which is specifically immunoreactive with at least one anti-polypeptide antigen antibody. Further, such a kit includes means for detecting the binding of said antibody to the antigen (e.g., the antibody may be conjugated to a fluorescent compound such as fluorescein or rhodamine which can be detected by flow cytometry). In specific embodiments, the kit may include a recombinantly produced or chemically synthesized polypeptide antigen. The polypeptide antigen of the kit may also be attached to a solid support.

In a more specific embodiment the detecting means of the above-described kit includes a solid support to which said polypeptide antigen is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the polypeptide antigen can be detected by binding of the said reporter-labeled antibody.

In an additional embodiment, the invention includes a diagnostic kit for use in screening serum containing antigens of the polypeptide of the invention. The diagnostic kit includes a substantially isolated antibody specifically immunoreactive with polypeptide or polynucleotide antigens, and means for detecting the binding of the polynucleotide or polypeptide antigen to the antibody. In one embodiment, the antibody is attached to a solid support. In a

specific embodiment, the antibody may be a monoclonal antibody. The detecting means of the kit may include a second, labeled monoclonal antibody.

Alternatively, or in addition, the detecting means may include a labeled, competing antigen.

5           In one diagnostic configuration, test serum is reacted with a solid phase reagent having a surface-bound antigen obtained by the methods of the present invention. After binding with specific antigen antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-antigen antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric, luminescent or colorimetric substrate (Sigma, St. Louis, MO).

10           The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plate or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Alternatively, streptavidin coated plates can be used 20 in conjunction with biotinylated antigen(s).

15           Thus, the invention provides an assay system or kit for carrying out this diagnostic method. The kit generally includes a support with surface- bound recombinant antigens, and a reporter-labeled anti-human antibody for detecting 25 surface-bound anti-antigen antibody.

## ***Immune System-Related Disorders***

### ***Diagnosis***

The present inventors have discovered that TNFR-6 alpha and TNFR-6 beta are expressed in hematopoietic and transformed tissues. For a number of immune system-related disorders, substantially altered (increased or decreased) levels of TNFR gene expression can be detected in immune system tissue or other cells or bodily fluids (e.g., sera and plasma) taken from an individual having such a disorder, relative to a "standard" TNFR gene expression level, that is, the TNFR expression level in immune system tissues or other cells or bodily fluids from an individual not having the immune system disorder. Thus, the invention provides a diagnostic method useful during diagnosis of an immune system disorder, which involves measuring the expression level of the gene encoding the TNFR protein in immune system tissue or other cells or body fluid from an individual and comparing the measured gene expression level with a standard TNFR gene expression level, whereby an increase or decrease in the gene expression level compared to the standard is indicative of an immune system disorder.

In particular, it is believed that certain tissues in mammals with cancer (e.g., colon, breast and lung cancers) have elevated copy numbers of TNFR genes and/or express significantly elevated levels of the TNFR protein and mRNA encoding the TNFR when compared to a corresponding "standard" level. Further, it is believed that elevated levels of the TNFR protein can be detected in certain cells or body fluids (e.g., sera and plasma) from mammals with such a cancer when compared to sera from mammals of the same species not having the cancer.

Thus, the invention provides a diagnostic method useful during diagnosis of an immune system disorder, including cancers which involves measuring the expression level of the gene encoding the TNFR protein in

immune system tissue or other cells or body fluid from an individual and comparing the measured gene expression level with a standard TNFR gene expression level, whereby an increase or decrease in the gene expression level compared to the standard is indicative of an immune system disorder.

5 Where a diagnosis of a disorder in the immune system including diagnosis of a tumor has already been made according to conventional methods, the present invention is useful as a prognostic indicator, whereby patients exhibiting depressed gene expression will experience a worse clinical outcome relative to patients expressing the gene at a level nearer the standard level.

10 By "assaying the expression level of the gene encoding a TNFR protein" is intended qualitatively or quantitatively measuring or estimating the level of the TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  protein or the level of the mRNA encoding the TNFR-6 $\alpha$  and/or TNFR-6 $\beta$  protein in a first biological sample either directly (e.g., by determining or estimating absolute protein level or mRNA level) or relatively (e.g., by comparing to the TNFR protein level or mRNA level in a second biological sample). Preferably, the TNFR protein level or mRNA level in the first biological sample is measured or estimated and compared to a standard TNFR protein level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having 15 the disorder or being determined by averaging levels from a population of individuals not having a disorder of the immune system. As will be appreciated in the art, once standard TNFR protein levels or mRNA levels are known, they can be used repeatedly as a standard for comparison.

20 By "biological sample" is intended any biological sample obtained from an individual, body fluid, cell line, tissue culture, or other source which contains TNFR protein or mRNA. As indicated, biological samples include 25 body fluids (such as sera, plasma, urine, synovial fluid and spinal fluid) which contain free extracellular domain(s) (or soluble form(s)) of a TNFR protein,

immune system tissue, and other tissue sources found to express complete TNFR, mature TNFR, or extracellular domain of a TNFR. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. Where the biological sample is to include mRNA, a tissue biopsy is the preferred source.

The invention also contemplates the use of a gene of the present invention for diagnosing mutations in a TNFR gene. For example, if a mutation is present in one of the genes of the present invention, conditions would result from a lack of production of the receptor polypeptides of the present invention. Further, mutations which enhance receptor polypeptide activity would lead to diseases associated with an over expression of the receptor polypeptide, e.g., cancer. Mutations in the genes can be detected by comparing the sequence of the defective gene with that of a normal one.

Subsequently one can verify that a mutant gene is associated with a disease condition or the susceptibility to a disease condition. That is, a mutant gene which leads to the underexpression of the receptor polypeptides of the present invention would be associated with an inability of TNFR to inhibit Fas ligand and/or AIM-II mediated apoptosis, and thereby result in irregular cell proliferation (e.g., tumor growth).

Other immune system disorders which may be diagnosed by the foregoing assays include, but are not limited to, hypersensitivity, allergy, infectious disease, graft-host disease, Immunodeficiency, autoimmune diseases and the like.

Individuals carrying mutations in the genes of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids used for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva and tissue biopsy among other tissues. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, *Nature*, 324:163-166 (1986)) prior to analysis. RNA or

cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid of the instant invention can be used to identify and analyze mutations in the human genes of the present invention. For example, deletions and insertions can be detected by a change in the size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA or alternatively, radiolabeled antisense DNA sequences of the present invention. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures. Such a diagnostic would be particularly useful for prenatal or even neonatal testing.

Sequence differences between the reference gene and "mutants" may be revealed by the direct DNA sequencing method. In addition, cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer used with double stranded PCR product or a single stranded template molecule generated by a modified PCR product. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

Sequence changes at the specific locations may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (for example, Cotton *et al.*, *PNAS*, 85:4397-4401 (1985)).

Assaying TNFR protein levels in a biological sample can occur using antibody-based techniques. For example, TNFR protein expression in tissues can be studied with classical immunohistological methods (Jalkanen, M., *et al.*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., *et al.*, *J. Cell. Biol.* 105:3087-3096 (1987)). Other antibody-based methods useful for detecting TNFR gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable

antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine ( $^{125}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{112}\text{In}$ ), and technetium ( $^{99\text{m}}\text{Tc}$ ), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

5 In addition to assaying TNFR protein levels in a biological sample obtained from an individual, TNFR proteins can also be detected *in vivo* by imaging. Antibody labels or markers for *in vivo* imaging of TNFR proteins include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable  
10 markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

15 A TNFR-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example,  $^{131}\text{I}$ ,  $^{112}\text{In}$ ,  $^{99\text{m}}\text{Tc}$ , ( $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{123}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{115\text{m}}\text{In}$ ,  $^{113\text{m}}\text{In}$ ,  $^{112}\text{In}$ ,  $^{111}\text{In}$ ), and technetium ( $^{99}\text{Tc}$ ,  $^{99\text{m}}\text{Tc}$ ), thallium ( $^{201}\text{Ti}$ ), gallium ( $^{68}\text{Ga}$ ,  $^{67}\text{Ga}$ ), palladium ( $^{103}\text{Pd}$ ), molybdenum ( $^{99}\text{Mo}$ ), xenon ( $^{133}\text{Xe}$ ), fluorine ( $^{18}\text{F}$ ),  $^{153}\text{Sm}$ ,  $^{177}\text{Lu}$ ,  $^{159}\text{Gd}$ ,  $^{149}\text{Pm}$ ,  $^{140}\text{La}$ ,  $^{175}\text{Yb}$ ,  $^{166}\text{Ho}$ ,  
20  $^{90}\text{Y}$ ,  $^{47}\text{Sc}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{142}\text{Pr}$ ,  $^{105}\text{Rh}$ ,  $^{97}\text{Ru}$ ), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously or intraperitoneally) into the mammal to be  
25 examined for immune system disorder. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of  $^{99\text{m}}\text{Tc}$ . The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain Neutrokin-alpha protein. *In vivo* tumor

imaging is described in S.W. Burchiel et al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments" (Chapter 13 in *Tumor Imaging: The Radiochemical Detection of Cancer*, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982)).

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### ***Treatment***

The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol.* 51:597-609 (1986), Cold Spring Harbor; Beutler, B., and Cerami, A., *Annu. Rev. Biochem.* 57:505-518 (1988); Old, L.J., *Sci. Am.* 258:59-75 (1988); Fiers, W., *FEBS Lett.* 285:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors.

TNFR-6 alpha and/or TNFR-6 beta polynucleotides and polypeptides of the invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of TNFR-6 alpha and/or TNFR-6 beta . TNFR-6 alpha and/or TNFR-6 beta polypeptides may be administered to a patient (e.g., mammal, preferably human) afflicted with such a disorder. Alternatively, a gene therapy approach may be applied to treat such disorders. Disclosure herein of TNFR-6 alpha and/or TNFR-6 beta nucleotide sequences permits the detection of defective TNFR-6 alpha and/or TNFR-6 beta genes, and the replacement thereof with normal TNFR-6 alpha and/or TNFR-6 beta -encoding genes. Defective genes may be detected in *in vitro* diagnostic assays, and by comparison of a TNFR-6 alpha and/or TNFR-6 beta nucleotide sequence disclosed herein with that of a

TNFR-6 alpha and/or TNFR-6 beta gene derived from a patient suspected of harboring a defect in this gene.

In another embodiment, the polypeptides of the present invention are used as a research tool for studying the biological effects that result from inhibiting Fas ligand/TNFR-6 alpha and/or TNFR-6 beta and/or AIM-II interactions on different cell types. TNFR-6 alpha and/or TNFR-6 beta polypeptides also may be employed in *in vitro* assays for detecting Fas ligand, AIM-II, or TNFR-6 alpha and/or TNFR-6 beta or the interactions thereof.

In another embodiment, a purified TNFR-6 alpha and/or TNFR-6 beta polypeptide of the invention is used to inhibit binding of Fas ligand and/or AIM-II to endogenous cell surface Fas ligand and/or AIM-II receptors. Certain ligands of the TNF family (of which Fas ligand and AIM-II are members) have been reported to bind to more than one distinct cell surface receptor protein. AIM-II likewise is believed to bind multiple cell surface proteins. By binding Fas ligand and/or AIM-II, soluble TNFR-6 alpha and/or TNFR-6 beta polypeptides of the present invention may be employed to inhibit the binding of Fas ligand and/or AIM-II not only to endogenous TNFR-6 alpha and/or TNFR-6 beta, but also to Fas ligand and AIM-II receptor proteins that are distinct from TNFR-6 alpha and/or TNFR-6 beta. Thus, in another embodiment, TNFR-6 alpha and/or TNFR-6 beta is used to inhibit a biological activity of Fas ligand and/or AIM-II, in *in vitro* or *in vivo* procedures. By inhibiting binding of Fas ligand and/or AIM-II to cell surface receptors, TNFR-6 alpha and/or TNFR-6 beta polypeptides of the invention also inhibit biological effects that result from the binding of Fas ligand and/or AIM-II to endogenous receptors. Various forms of TNFR-6 alpha and/or TNFR-6 beta may be employed, including, for example, the above-described TNFR-6 alpha and/or TNFR-6 beta fragments, derivatives, and variants that are capable of binding Fas ligand and/or AIM-II. In a preferred embodiment, a soluble TNFR-6 alpha and/or TNFR-6 beta polypeptide of the invention is administered to

inhibit a biological activity of Fas ligand and/or AIM-II, e.g., to inhibit Fas ligand-mediated and/or AIM-II-mediated apoptosis of cells susceptible to such apoptosis.

In a further embodiment, a TNFR-6 alpha and/or TNFR-6 beta polypeptide of the invention is administered to a mammal to treat a Fas ligand-mediated and/or AIM-II-mediated disorder. Such Fas ligand-mediated and/or AIM-II-mediated (e.g., a human) disorders include conditions caused (directly or indirectly) or exacerbated by Fas ligand and/or AIM-II.

Cells which express a TNFR polypeptide and have a potent cellular response to TNFR-6 $\alpha$  and TNFR-6 $\beta$  ligands include lymphocytes, endothelial cells, keratinocytes, and prostate tissue. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis.

Additionally, as described herein, TNFR polypeptides of the invention bind Fas ligand and AIM-II and consequently block Fas ligand and AIM-II mediated apoptosis. Apoptosis-programmed cell death is a physiological mechanism involved in the deletion of B and/or T lymphocytes of the immune system, and its disregulation can lead to a number of different pathogenic processes (J.C. Ameisen AIDS 8:1197-1213 (1994); P.H. Krammer *et al.*, Curr. Opin. Immunol. 6:279-289 (1994)).

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma,

osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Grave's disease, Hashimoto's thyroiditis, autoimmune diabetes, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis (e.g., proliferative glomerulonephritis), autoimmune gastritis, autoimmune thrombocytopenic purpura, and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation, graft vs. host disease (acute and/or chronic), acute graft rejection, and chronic graft rejection. In preferred embodiments, TNFR polynucleotides, polypeptides, and/or antagonists of the invention are used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above.

Additional diseases or conditions associated with increased cell survival include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endothelisarcoma, lymphangiosarcoma, lymphangioendothelisarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary

carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, 5 medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendrogioma, menangioma, melanoma, neuroblastoma, and retinoblastoma.

Diseases associated with increased apoptosis include AIDS; 10 neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration and brain tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Grave's disease Hashimoto's thyroiditis, autoimmune diabetes, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus, immune-related glomerulonephritis (e.g., proliferative glomerulonephritis), autoimmune gastritis, thrombocytopenic purpura, and rheumatoid arthritis) 15 myelodysplastic syndromes (such as aplastic anemia), graft vs. host disease (acute and/or chronic), ischemic injury (such as ischemic cardiac injury and that caused by myocardial infarction, stroke and reperfusion injury), liver 20 injury or disease (e.g., hepatitis related liver injury, cirrhosis, ischemia/reperfusion injury, cholestasis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, ulcerative colitis, cachexia and anorexia. In preferred embodiments, TNFR 25 polynucleotides, polypeptides and/or agonists are used to treat or prevent the diseases and disorders listed above.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat and/or prevent glomerulonephritis. In a further embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat and/or prevent chronic glomerulonephritis

and/or cell/tissue damage (e.g., glomerular cell death) and/or medical conditions associated with this disease. In a further nonexclusive embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat and/or prevent proliferative glomerulonephritis and/or cell/tissue damage (e.g., glomerular cell death) and/or medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used treat or prevent biliary cirrhosis and/or medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used treat or prevent disease, such as, for example, alcoholic liver disease and/or medical conditions associated with this disease (e.g., cirrhosis).

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat and/or prevent graft vs host disease. In a specific embodiment TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat (e.g., reduce) or prevent tissue or cell damage or destruction (e.g., lymphoid cell depletion associated with graft vs host disease) and/or other medical conditions associated with this disease. In another non exclusive specific embodiment, the TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat (e.g., reduce) and/or prevent diarrhea during graft vs host disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention are used to treat and/or prevent Sjogren's disease and/or to reduce tissue/cell damage or destruction (e.g., damage or destruction of salivary and/or lacrimal tissues) and/or other medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat and/or prevent multiple sclerosis and/or to reduce tissue damage or destruction (such as, for example,

neurological tissue (e.g., CNS tissue) damage or destruction) and/or lesions or other medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists, including antibody and antibody fragments, of the invention are used to treat and/or prevent Alzheimer's disease and/or to reduce tissue damage or destruction (e.g., damage or destruction of neurological tissue or cells) and/or medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat, prevent Parkinson's disease and/or to reduce tissue damage or destruction (e.g., damage or destruction of neurological tissue or cells, such as, for example neuronal cells) and/or medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used before, during, immediately after, and/or after a stroke to treat, prevent, or reduce damage of cells or tissue (such as, for example, neurological tissue) and/or medical conditions associated with stroke.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat, prevent, or reduce ischemic injury (such as, for example, ischemic cardiac injury) and/or medical conditions associated with ischemic injury. In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used before, during, immediately after, and/or after a heart attack to treat, prevent, or reduce ischemic cardiac injury.

In another specific embodiment, TNFR polynucleotides, polypeptides, and/or agonists of the invention are used to treat or prevent myelodysplastic syndromes (MDS) and/or medical conditions associated with MDS.

In another specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to increase circulating blood cell numbers in patients suffering from cytopenia, lymphopenia and/or anemia.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat and/or prevent Hashimoto's thyroiditis and/or to reduce destruction or damage of tissue or cells (e.g., thyroid gland) and/or to treat or prevent medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat (e.g., reduce) and/or prevent autoimmune gastritis and/or medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to treat and/or prevent ulcerative colitis and/or cell/tissue damage (e.g., ulceration in the colon) and/or medical conditions associated with this disease.

In a specific embodiment, TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention are used to treat and/or prevent rheumatoid arthritis and/or medical conditions associated with this disease.

Additionally, a number of cancers secrete FasL which binds Fas positive T cells and kills them. Any cancer which expresses FasL could therefore be a target for treatment by TNFR and TNFR agonists of the invention. Such cancers include, but are not limited to, malignant myeloma, leukemia and lymphoma.

Many of the pathologies associated with HIV are mediated by apoptosis, including HIV-induced nephropathy and HIV encephalitis. Thus, in additional preferred embodiments, TNFR polynucleotides, polypeptides, and/or TNFR agonists of the invention are used to treat or prevent AIDS and pathologies associated with AIDS. Another embodiment of the present invention is directed to the use of TNFR-6 alpha and/or TNFR-6 beta to reduce Fas ligand and/or AIM-II-mediated death of T cells in HIV-infected patients.

The state of Immunodeficiency that defines AIDS is secondary to a decrease in the number and function of CD4<sup>+</sup> T-lymphocytes. Recent reports estimate the daily loss of CD4<sup>+</sup> T cells to be between 3.5 X 10<sup>7</sup> and 2 X 10<sup>9</sup> cells (Wei X., *et al.*, *Nature* 373:117-122 (1995)). One cause of CD4<sup>+</sup> T cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis (see, for example, Meyaard *et al.*, *Science* 257:217-219, (1992); Groux *et al.*, *J Exp. Med.*, 175:331, (1992); and Oyaizu *et al.*, in *Cell Activation and Apoptosis in HIV Infection*, Andrieu and Lu, Eds., Plenum Press, New York, 1995, pp. 101-114). Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (Ameisen, J.C., *AIDS* 8:1197-1213 (1994) ; Finkel, T.H., and Banda, N.K., *Curr. Opin. Immunol.* 6:605-615(1995); Muro-Cacho, C.A. *et al.*, *J. Immunol.* 154:5555-5566 (1995)). Furthermore, apoptosis and CD4<sup>+</sup> T-lymphocyte depletion is tightly correlated in different animal models of AIDS (Brunner, T., *et al.*, *Nature* 373:441-444 (1995); Gougeon, M.L., *et al.*, *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)) and, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (Gougeon, M.L. *et al.*, *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)). Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the Fas Ligand. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of U937 cells with HIV results in the *de novo* expression of Fas ligand and that Fas ligand mediates HIV-induced apoptosis (Badley, A.D. *et al.*, *J. Virol.* 70:199-206 (1996)). Further the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes (Badley, A.D *et al.*, *J. Virol.* 70:199-206 (1996)). Further, additional studies have implicated Fas-mediated

apoptosis in loss of T cells in HIV individuals (Katsikis *et al.*, *J. Exp. Med.* 181:2029-2036, 1995).

Thus, by the invention, a method for treating HIV<sup>+</sup> individuals is provided which involves administering TNFR and/or TNFR agonists of the present invention to reduce selective killing of CD4 T-lymphocytes. Modes of administration and dosages are discussed in detail below.

It is also possible that T cell apoptosis occurs through multiple mechanisms. Further at least some of the T cell death seen in HIV patients may be mediated by AIM-II. While not wishing to be bound by theory, such Fas ligand and/or AIM-II-mediated T cell death is believed to occur through the mechanism known as activation-induced cell death (AICD).

Activated human T cells are induced to undergo programmed cell death (apoptosis) upon triggering through the CD3/T cell receptor complex, a process termed activated-induced cell death (AICD). AICD of CD4 T cells isolated from HIV-Infected asymptomatic individuals has been reported (Groux *et al.*, *supra*). Thus, AICD may play a role in the depletion of CD4+ T cells and the progression to AIDS in HIV-infected individuals. Thus, the present invention provides a method of inhibiting Fas ligand-mediated and/or AIM-II-mediated T cell death in HIV patients, comprising administering a TNFR-6 alpha and/or TNFR-6 beta polypeptide of the invention to the patients. In one embodiment, the patient is asymptomatic when treatment with TNFR-6 alpha and/or TNFR-6 beta commences. If desired, prior to treatment, peripheral blood T cells may be extracted from an HIV patient, and tested for susceptibility to Fas ligand-mediated and/or AIM-II-mediated cell death by conventional procedures. In one embodiment, a patient's blood or plasma is contacted with TNFR-6 alpha and/or TNFR-6 beta *ex vivo*. The TNFR-6 alpha and/or TNFR-6 beta may be bound to a suitable chromatography matrix known in the art by conventional procedures. The patient's blood or plasma flows through a chromatography column containing

TNFR-6 alpha and/or TNFR-6 beta polypeptides of the invention bound to the matrix, before being returned to the patient. The immobilized TNFR-6 alpha and/or TNFR-6 beta binds Fas ligand and/or AIM-II, thus removing Fas ligand and/or AIM-II protein from the patient's blood.

5           In additional embodiments a TNFR-6 alpha and/or TNFR-6 beta polypeptide of the invention may be administered in combination with other inhibitors of T cell apoptosis. For example, at least some of the T cell death seen in HIV patients is believed to be mediated by TRAIL (International application publication number WO 97/01633 hereby incorporated by reference). Thus, for example, a patient susceptible to both Fas ligand mediated and TRAIL mediated T cell death may be treated with both an agent that blocks TRAIL/TRAIL-receptor interactions and an agent that blocks Fas-ligand/Fas interactions. Suitable agents that may be administered with the polynucleotides and/or polypeptides of the invention to block binding of TRAIL to TRAIL receptors include, but are not limited to, soluble TRAIL receptor polypeptides (e.g., a soluble form of OPG, DR4 (International application publication number WO 98/32856); TR5 (International application publication number WO 98/30693); DR5 (International application publication number WO 98/41629); TR10 (International application publication number WO 98/54202)); multimeric forms of soluble TRAIL receptor polypeptides; and TRAIL receptor antibodies that bind the TRAIL receptor without transducing the biological signal that results in apoptosis, anti-TRAIL antibodies that block binding of TRAIL to one or more TRAIL receptors, and muteins of TRAIL that bind TRAIL receptors but do not transduce the biological signal that results in apoptosis. Preferably, the antibodies employed according to this method are monoclonal antibodies.

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Suitable agents, which also block binding of Fas-ligand to Fas that may be administered with the polynucleotides and polypeptides of the present invention include, but are not limited to, soluble Fas polypeptides; multimeric

forms of soluble Fas polypeptides (e.g., dimers of sFas/Fc); anti-Fas antibodies that bind Fas without transducing the biological signal that results in apoptosis; anti-Fas-ligand antibodies that block binding of Fas-ligand to Fas; and muteins of Fas-ligand that bind Fas but do not transduce the biological signal that results in apoptosis. Examples of suitable agents for blocking Fas-L/Fas interactions, including blocking anti-Fas monoclonal antibodies, are described in International application publication number WO 95/10540, hereby incorporated by reference.

Suitable agents that may be administered with the polynucleotides and/or polypeptides of the invention to block binding of AIM-II to AIM-II receptors include, but are not limited to, soluble AIM-II receptor polypeptides (e.g., a soluble form of TR2 (International application publication number WO 96/34095); LT beta receptor; and TR8 (International application publication number WO 98/54201)); multimeric forms of soluble AIM-II receptor polypeptides; and AIM-II receptor antibodies that bind the AIM-II receptor without transducing the biological signal that results in apoptosis, anti-AIM-II antibodies that block binding of AIM-II to one or more AIM-II receptors, and muteins of AIM-II that bind AIM-II receptors but do not transduce the biological signal that results in apoptosis. Preferably, the antibodies employed according to this method are monoclonal antibodies.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already

been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage.

Antagonists of the present invention are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the TNFR polypeptide, and thereby are susceptible to compounds which enhance TNFR activity. Thus, the present invention further provides a method for creating immune privileged tissues. Antagonist of the invention can further be used in the treatment of Inflammatory Bowel-Disease.

TNFR polynucleotides, polypeptides, and agonists of the invention may also be used to suppress immune responses. In one embodiment, the TNFR polynucleotides, polypeptides, and agonists of the invention are used to minimize untoward effects associated with transplantation. In a specific embodiment, the TNFR polynucleotides, polypeptides, and agonists of the invention are used to suppress Fas mediated immune responses (e.g., in a manner similar to an immunosuppressant such as, for example, rapamycin or cyclosporin). In another specific embodiment, the TNFR polynucleotides, polypeptides, and agonists of the invention are used to suppress AIM-II mediated immune responses.

Additionally, both graft rejection and graft vs. host disease are in part triggered by apoptosis. Accordingly, an additional preferred embodiment, TNFR polynucleotides, polypeptides, and/or TNFR agonists of the invention are used to treat and prevent and/or reduce graft rejection. In a further preferred embodiment, TNFR polynucleotides, polypeptides, and/or TNFR agonists of the invention are used to treat and prevent and/or reduce graft vs. host disease.

Additionally, TNFR-6 alpha and/or TNFR-6 beta polypeptides, polynucleotides, and/or agonists may be used to treat or prevent graft rejection (e.g., xenograft and allograft rejection (e.g, acute allograft rejection))

and/or medical conditions associated with graft rejection. In a specific embodiment, TNFR-6 alpha and/or TNFR-6 beta polypeptides, polynucleotides, and/or agonists of the invention are used to treat or prevent acute allograft rejection and/or medical conditions associated with acute allograft rejection. In a further specific embodiment, TNFR-6 alpha and/or TNFR-6 beta polypeptides, polynucleotides, and/or agonists of the invention are used to treat or prevent acute allograft rejection of a kidney and/or medical conditions associated with acute allograft rejection of a kidney.

Fas ligand is a type II membrane protein that induces apoptosis by binding to Fas. Fas ligand is expressed in activated T cells, and works as an effector of cytotoxic lymphocytes. Molecular and genetic analysis of Fas and Fas ligand have indicated that mouse lymphoproliferation mutation (*lpr*) and generalized lymphoproliferative disease (*gld*) are mutations of Fas and Fas ligand respectively. The *lpr* of *gld* mice develop lymphadenopathy, and suffer from autoimmune disease. Based on these phenotypes and other studies, it is believed that the Fas system is involved in the apoptotic process during T-cell development, specifically peripheral clonal deletion or activation-induced suicide of mature T cells. In addition to the activated lymphocytes, Fas is expressed in the liver, heart and lung. Administration of agonistic anti-Fas antibody into mice has been shown to induce apoptosis in the liver and to quickly kill the mice, causing liver damage. These findings indicate that the Fas system plays a role not only in the physiological process of lymphocyte development, but also in the cytotoxic T-lymphocyte-mediated disease such as fulminant hepatitis and/or hepatitis resulting from viral infection or toxic agents. As discussed herein, TNFR-6 alpha and/or TNFR-6 beta binds Fas ligand, and thus functions as an antagonist of Fas-ligand mediated activity. Accordingly, the TNFR-6 alpha and/or TNFR-6 beta polypeptides and/or polynucleotides of the invention, and/or agonists thereof, may be used to treat or prevent lymphoproliferative disorders (e.g., lymphadenopathy and others

described herein), autoimmune disorders (e.g., autoimmune diabetes, systemic lupus erythematosus, Grave's disease, Hashimoto's thyroiditis, immune-related glomerulonephritis, autoimmune gastritis, autoimmune thrombocytopenic purpura, multiple sclerosis, rheumatoid arthritis, and others described herein), and/or liver disease (e.g., acute and chronic hepatitis, and cirrhosis).

In a specific embodiment TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention is used to treat or prevent hepatitis and/or tissue/cell damage or destruction and/or medical conditions associated with hepatitis. In a specific embodiment TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention is used to treat or prevent fulminant hepatitis and/or medical conditions associated with fulminant hepatitis.

In a specific embodiment TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention is used to treat or prevent systemic lupus erythematosus (SLE) and/or tissue/cell damage or destruction and/or medical conditions associated with SLE. In a further specific embodiment, TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention are used to treat or prevent skin lesions in SLE patients.

In a specific embodiment, TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention is used to treat or prevent insulin-dependent diabetes mellitus and/or tissue/cell damage or destruction and/or medical conditions associated with insulin-dependent diabetes mellitus. In a further specific embodiment, TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention are prior to, during, or immediately after the onset of diabetes to reduce or prevent damage to islet cells and/or to reduce exogenous insulin requirement.

In a specific embodiment TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention is used to treat or prevent toxic

epidermal necrolysis (TEN) and/or tissue/cell damage or destruction, and/or medical conditions associated with TEN. In a further specific embodiment, TNFR polynucleotides, polypeptides, and/or agonists or antagonists of the invention is used to treat or prevent Lyell's syndrome.

5            Hepatitis virus (e.g., Hepatitis B virus and Hepatitis C virus) is a major causative agent of chronic liver disease. In Hepatitis infection, Fas expression in hepatocytes is up-regulated in accordance with the severity of liver inflammation. When Hepatitis virus-specific T cells migrate into hepatocytes and recognize the viral antigen via the T cell receptor, they become activated and express Fas ligand that can transduce the apoptotic death signal to Fas-bearing hepatocytes. Thus, the Fas system plays an important role in liver cell injury by viral hepatitis. Accordingly, in specific 10          embodiments, the TNFR-6 alpha and/or TNFR-6 beta polypeptides and/or polynucleotides of the invention and/or agonists or antagonists thereof, are used to treat or prevent hepatitis resulting from viral infection (e.g., infection 15          resulting form Hepatitis B virus or Hepatitis C virus infection). In one embodiment, a patient's blood or plasma is contacted with TNFR-6 alpha and/or TNFR-6 beta polypeptides of the invention *ex vivo*. The TNFR-6 alpha and/or TNFR-6 beta may be bound to a suitable chromatography matrix 20          by conventional procedures. According to this embodiment, the patient's blood or plasma flows through a chromatography column containing TNFR-6 alpha and/or TNFR-6 beta bound to the matrix, before being returned to the patient. The immobilized TNFR-6 alpha and/or TNFR-6 beta binds Fas-ligand, thus removing Fas-ligand protein from the patient's blood. 25

In a specific embodiment, TNFR-6 alpha and/or TNFR-6 beta polypeptides, polynucleotides, and/or agonists or antagonists of the invention may be used to treat or prevent renal failure (e.g., chronic renal failure), and/or tissue/cell damage or destruction (e.g., tubular epithelial cell deletion) and/or medical conditions associated with renal failure.

In a specific embodiment, TNFR-6 alpha and/or TNFR-6 beta polypeptides, polynucleotides, and/or agonists or antagonists of the invention may be used to regulate (i.e., stimulate or inhibit) bone growth. In specific embodiments TNFR-6 alpha and/or TNFR-6 beta polypeptides, 5 polynucleotides, and/or agonists or antagonists of the invention are used to stimulate bone growth. Specific diseases or conditions that may be treated or prevented with the compositions of the invention include, but are not limited to, bone fractures, and defects, and disorders which result in weakened bones such as osteoporosis, osteomalacia, and age-related loss of bone mass.

10 TNFR-6 alpha and/or TNFR-6 beta polypeptides or polynucleotides encoding TNFR-6 alpha and/or TNFR-6 beta of the invention, and/or agonists or antagonists thereof may be used to treat or prevent cardiovascular disorders, including peripheral artery disease, such as limb ischemia.

15 Cardiovascular disorders include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogy of Fallot, ventricular heart septal defects.

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25 Cardiovascular disorders also include heart disease, such as atherosclerosis, arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure (e.g., chronic congestive heart failure), congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema,

heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiectomy syndrome, pulmonary fibrosis, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

In a specific embodiment, TNFR-6 alpha and/or TNFR-6 beta polynucleotides, polypeptides, or agonists of the invention may be used to treat and/or prevent chronic congestive heart failure and/or medical conditions associated chronic congestive heart failure.

In another specific embodiment, TNFR-6 alpha and/or TNFR-6 beta polynucleotides, polypeptides, or agonists of the invention may be used to treat and/or prevent pulmonary injury or disease (e.g., pulmonary fibrosis and chronic obstructive pulmonary diseases, such as, for example, emphysema and chronic bronchitis), and/or tissue/cell damage or destruction (e.g., alveolar wall and/or bronchiolar wall destruction) and/or medical conditions associated with pulmonary injury or disease.

Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus syndrome, tachycardias, and ventricular fibrillation.

Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

Heart valve disease include aortic valve insufficiency, aortic valve stenosis, hear murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

Cardiovascular diseases also include vascular diseases such as aneurysms, angiodyplasia, angiomas, bacillary angiomas, Hippel-Lindau Disease, Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis, pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, atacia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

Arterial occlusive diseases include arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

5 Cerebrovascular disorders include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subarachnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

10 15 Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromboembolisms. Thromboses include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

20 Ischemia includes cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

25 In one embodiment, TNFR-6 alpha and/or TNFR-6 beta polypeptides, polynucleotides and/or agonists or antagonists of the invention are used to treat or prevent thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H.C., *Semin. Hematol.* 24:71 (1987); Thompson *et al.*, *Blood* 80:1890 (1992)). Increasing

TTP-associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok *et al.*, *Am. J. Hematol.* 50:84 (1995)). Plasma from patients afflicted with TTP (including HIV+ and HIV- patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence *et al.*, *Blood* 87:3245 (1996)). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. An anti-Fas blocking antibody has been shown to reduce TTP plasma-mediated apoptosis of microvascular endothelial cells (Lawrence *et al.*, *Blood* 87:3245 (1996); hereby incorporated by reference).

Accordingly, Fas ligand present in the serum of TTP patients is likely to play a role in inducing apoptosis of microvascular endothelial cells. Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J.L., *Lancet*, 343:393, (1994); Melnyk *et al.*, (*Arch. Intern. Med.*, 155:2077, (1995); Thompson *et al.*, *supra*). Thus, in one embodiment, the invention is directed to use of TNFR-6 alpha and/or TNFR-6 beta to treat or prevent the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS. In another embodiment, conditions characterized by clotting of small blood vessels may be treated using TNFR-6 alpha and/or TNFR-6 beta polypeptides and/or polynucleotides of the invention. Such conditions include, but are not limited to, those described herein. For example, cardiac problems seen in about 5-10% of pediatric AIDS patients are believed to involve clotting of small blood vessels. Breakdown of the microvasculature in the heart has been reported in multiple sclerosis patients. As a further example, treatment of systemic lupus erythematosus (SLE) is contemplated. In one embodiment, a patient's blood or plasma is contacted with TNFR-6 alpha and/or TNFR-6 beta polypeptides of the invention *ex vivo*. The TNFR-6 alpha and/or TNFR-6 beta may be bound to a suitable chromatography matrix using techniques known in the art. According

to this embodiment, the patient's blood or plasma flows through a chromatography column containing TNFR-6 alpha and/or TNFR-6 beta bound to the matrix, before being returned to the patient. The immobilized TNFR-6 alpha and/or TNFR-6 beta binds Fas ligand and/or AIM-II, thus removing Fas ligand protein from the patient's blood. Alternatively, TNFR-6 alpha and/or TNFR-6 beta may be administered *in vivo* to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a TNFR-6 alpha and/or TNFR-6 beta polynucleotide or polypeptide of the invention is administered to the patient. Thus, the present invention provides a method for treating a thrombotic microangiopathy, involving use of an effective amount of a TNFR-6 alpha and/or TNFR-6 beta polypeptide of the invention. A TNFR-6 alpha and/or TNFR-6 beta polypeptide may be employed in *in vivo* or *ex vivo* procedures, to inhibit Fas ligand-mediated and/or AIM-II-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

TNFR-6 alpha and/or TNFR-6 beta polypeptides and polynucleotides of the invention may be employed in conjunction with other agents useful in treating a particular disorder. For example, in an *in vitro* study reported by Laurence *et al.* (*Blood* 87:3245, 1996), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate. Thus, a patient may be treated in combination with an additional agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells such as, for example, an agent described above. In one embodiment, TNFR-6 alpha and/or TNFR-6 beta polypeptides of the invention and an anti-FAS blocking antibody are administered to a patient afflicted with a disorder characterized by thrombotic microangiopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in International Application publication number WO 95/10540, hereby incorporated by reference.

The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate. Rastinejad *et al.*, *Cell* 56:345-355 (1989). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases. A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye disorders, and psoriasis. See, e.g., reviews by Moses *et al.*, *Biotech.* 9:630-634 (1991); Folkman *et al.*, *N. Engl. J. Med.*, 333:1757-1763 (1995); Auerbach *et al.*, *J. Microvasc. Res.* 29:401-411 (1985); Folkman, Advances in Cancer Research, eds. Klein and Weinhouse, Academic Press, New York, pp. 175-203 (1985); Patz, *Am. J. Ophthalmol.* 94:715-743 (1982); and Folkman *et al.*, *Science* 221:719-725 (1983). In a number of pathological conditions, the process of angiogenesis contributes to the disease state. For example, significant data have accumulated which suggest that the growth of solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, *Science* 235:442-447 (1987).

The present invention provides for treatment of diseases or disorders associated with neovascularization by administration of the TNFR-6 alpha and/or TNFR-6 beta polynucleotides and/or polypeptides of the invention. Malignant and metastatic conditions which can be treated with the polynucleotides and polypeptides of the invention include, but are not limited to, malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman *et al.*, *Medicine*, 2d Ed., J. B. Lippincott Co., Philadelphia (1985));

Ocular disorders associated with neovascularization which can be treated with the TNFR-6 alpha and/or TNFR-6 beta polynucleotides and polypeptides of the present invention (including TNFR agonists and/or antagonists) include, but are not limited to: neovascular glaucoma, diabetic retinopathy, retinoblastoma, retrobulbar fibroplasia, uveitis, retinopathy of prematurity macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization. See, e.g., reviews by Waltman *et al.*, *Am. J. Ophthal.* 85:704-710 (1978) and Gartner *et al.*, *Surv. Ophthalmol.* 22:291-312 (1978).

In another embodiment, TNFR-6 alpha and/or TNFR-6 beta polypeptides, polynucleotides and/or agonists or antagonists of the invention are used to stimulate differentiation and/or survival of photoreceptor cells and/or to treat or prevent diseases, disorders, or conditions associated with decreased number, differentiation and/or survival of photoreceptor cells.

Additionally, disorders which can be treated with the TNFR-6 alpha and/or TNFR-6 beta polynucleotides and polypeptides of the present invention (including TNFR agonist and/or antagonists) include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

In additional embodiments, TNFR-6 alpha and/or TNFR-6 beta polynucleotides, polynucleotides and/or other compositions of the invention (e.g., anti-TNFR-6 alpha and/or anti-TNFR-6 beta antibodies) are used to treat or prevent diseases or conditions associated with allergy and/or inflammation.

In a specific embodiment TNFR polynucleotides, polypeptides and/or agonists or antagonists thereof may be used to treat or prevent thyroid-

associated ophthalmopathy and/or tissue/cell damage or destruction, and/or medical conditions associated with thyroid-associated ophthalmopathy.

In a specific embodiment, TNFR polynucleotides, polypeptides, or agonists of the invention are used to prolong protein expression after gene therapy by inhibiting or reducing elimination of transgene expressing cells.

In further embodiments, the TNFR-6 alpha and/or TNFR-6 beta polynucleotides and/or polynucleotides, and/or agonists or antagonists thereof, are used to promote wound healing.

Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in the diagnosis and treatment or prevention of a wide range of diseases and/or conditions. Such diseases and conditions include, but are not limited to, cancer (e.g., immune cell related cancers, breast cancer, prostate cancer, ovarian cancer, follicular lymphoma, cancer associated with mutation or alteration of p53, brain tumor, bladder cancer, uterocervical cancer, colon cancer, colorectal cancer, non-small cell carcinoma of the lung, small cell carcinoma of the lung, stomach cancer, etc.), lymphoproliferative disorders (e.g., lymphadenopathy), microbial (e.g., viral, bacterial, etc.) infection (e.g., HIV-1 infection, HIV-2 infection, herpesvirus infection (including, but not limited to, HSV-1, HSV-2, CMV, VZV, HHV-6, HHV-7, EBV), adenovirus infection, poxvirus infection, human papilloma virus infection, hepatitis infection (e.g., HAV, HBV, HCV, etc.), *Helicobacter pylori* infection, invasive Staphylococcia, etc.), parasitic infection, nephritis, bone disease (e.g., osteoporosis), atherosclerosis, pain, cardiovascular disorders (e.g., neovascularization, hypovascularization or reduced circulation (e.g., ischemic disease (e.g., myocardial infarction, stroke, etc.))), AIDS, allergy, inflammation, neurodegenerative disease (e.g., Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, pigmentary retinitis, cerebellar degeneration, etc.), graft rejection (acute and chronic), graft vs. host disease, diseases due to osteomyelodysplasia (e.g., aplastic anemia, etc.), joint

tissue destruction in rheumatism, liver disease (e.g., acute and chronic hepatitis, liver injury, and cirrhosis), autoimmune disease (e.g., multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, immune complex glomerulonephritis, autoimmune diabetes, autoimmune  
5 thombocytopenic purpura, Grave's disease, Hashimoto's thyroiditis, etc.), cardiomyopathy (e.g., dilated cardiomyopathy), diabetes, diabetic complications (e.g., diabetic nephropathy, diabetic neuropathy, diabetic retinopathy), influenza, asthma, psoriasis, glomerulonephritis, septic shock, and ulcerative colitis.

10 Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in promoting angiogenesis, regulating hematopoiesis and wound healing (e.g., wounds, burns, and bone fractures).

15 Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are also useful as an adjuvant to enhance immune responsiveness to specific antigen, anti-viral immune responses.

More generally, polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in regulating (i.e., elevating or reducing) immune response. For example, polynucleotides and/or polypeptides of the invention may be useful in preparation or recovery from surgery, trauma, radiation therapy, chemotherapy, and transplantation, or may be used to boost immune response and/or recovery in the elderly and immunocompromised individuals. Alternatively, polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful as immunosuppressive agents, for example in the treatment or prevention of autoimmune disorders. In specific embodiments, polynucleotides and/or polypeptides of the invention are used to treat or prevent chronic inflammatory, allergic or autoimmune conditions, such as those described herein or are otherwise known in the art.  
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In one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a patient (preferably a human) a TNFR antagonists (e.g., an anti-TNFR antibody or TNFR polypeptide fragment). Preferably, the TNFR antagonist is administered to treat a disease or condition wherein increased cell survival is exhibited. Antagonists of the invention include soluble forms of TNFR and monoclonal antibodies directed against the TNFR polypeptide.

By "antagonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "agonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can inhibit or enhance apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in International application publication number WO 92/01810, published February 6, 1992. Such an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in *Science* 246:181-296 (October 1989). For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the

present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

Another method involves screening for compounds which inhibit activation of the receptor polypeptide of the present invention antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA encoding the receptor such that the cell expresses the receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity of the receptors. If the compound binds to the receptor as determined by a reduction of labeled ligand which binds to the receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307(1992).

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the TNFR polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the TNFR polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor, neurotransmitters (such as glutamate, dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonists include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and amyloid peptide. (*Science* 267:1457-1458 (1995)). Further preferred agonists include polyclonal and monoclonal antibodies raised against the TNFR polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in Tartaglia, L.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 88:9292-9296 (1991); and Tartaglia, L.A., and Goeddel, D.V., *J.*

*Biol. Chem.* 267 (7):4304-4307 (1992) See, also, International application publication number WO 94/09137.

Antagonists according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus 5 *ElB*, Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRF1*, *LMP-1*, African swine fever virus *LMW5-HL*, and Herpesvirus yl 34.5), calpain inhibitors, cysteine protease inhibitors, and tumor promoters (such as PMA, Phenobarbital, and -Hexachlorocyclohexane). Other 10 antagonists include polyclonal and monoclonal antagonist antibodies raised against the TNFR polypeptides or a fragment thereof. Such antagonist antibodies raised against a TNF-family receptor are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307 (1992) and 15 Tartaglia, L.A. et al., *Cell* 73:213-216 (1993). See, also, International application publication number WO 94/09137.

In specific embodiments, antagonists according to the present invention are nucleic acids corresponding to the sequences contained in TNFR, or the complementary strand thereof, and/or to nucleotide sequences contained in the deposited clones (ATCC Deposit Nos. 97810 and 97809). In one 20 embodiment, antisense sequence is generated internally by the organism, in another embodiment, the antisense sequence is separately administered (see, for example, O'Connor, J., *Neurochem.* 56:560 (1991) and 25 Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed for example, in Okano, J., *Neurochem.* 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance, Lee et al., *Nucleic Acids Research* 6:3073 (1979);

Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1300 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into receptor polypeptide.

In one embodiment, the TNFR antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the TNFR antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding TNFR, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, *Nature* 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell* 22:787-797 (1980), the herpes thymidine promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, *Nature* 296:39-42 (1982)), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a TNFR gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded TNFR antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with a TNFR RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., *Nature* 372:333-335 (1994). Thus, oligonucleotides complementary to either the 5'- or 3'- non-translated, non-coding regions of the TNFR shown in Figures 1 and <sup>2A-B</sup> ~~1A~~ could be used in an antisense approach to inhibit translation of endogenous TNFR mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of TNFR mRNA, antisense nucleic acids should be at least six nucleotides in length, and are

preferably oligonucleotides ranging from 6 to about 50 nucleotides in length.

In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

The polynucleotides of the invention can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, e.g., Letsinger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556 (1989); Lemaitre *et al.*, *Proc. Natl. Acad. Sci.* 84:648-652 (1987); PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol *et al.*, *BioTechniques* 6:958-976 (1988)) or intercalating agents. (See, e.g., Zon, *Pharm. Res.* 5:539-549 (1988)). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine,

5-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an  $\alpha$ -anomeric oligonucleotide. An  $\alpha$ -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gautier *et al.*, *Nucl. Acids Res.* 15:6625-6641 (1987)). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue *et al.*, *Nucl. Acids Res.* 15:6131-6148 (1987)), or a chimeric RNA-DNA analogue (Inoue *et al.*, *FEBS Lett.* 215:327-330 (1987)).

Polynucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (*Nucl. Acids Res.* 16:3209 (1988)), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85:7448-7451 (1988)), etc.

While antisense nucleotides complementary to the TNFR coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred.

Potential antagonists according to the invention also include catalytic RNA, or a ribozyme (See, e.g., International application publication number WO 90/11364, published October 4, 1990; Sarver *et al*, *Science* 247:1222-1225 (1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy TNFR mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, *Nature* 334:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequence of TNFR-6 $\alpha$  (Figure 1, SEQ ID NO:1) and TNFR-6 $\beta$  (Figure 2, SEQ ID NO:3). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the TNFR mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

As in the antisense approach, the ribozymes of the invention can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express TNFR *in vivo*. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous TNFR messages

and inhibit translation. Since ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous gene expression can also be reduced by inactivating or "knocking out" the TNFR gene and/or its promoter using targeted homologous recombination. (E.g., see Smithies *et al.*, *Nature* 317:230-234 (1985); Thomas & Capecchi, *Cell* 51:503-512 (1987); Thompson *et al.*, *Cell* 5:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the invention (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous polynucleotide sequence (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express polypeptides of the invention *in vivo*. In another embodiment, techniques known in the art are used to generate knockouts in cells that contain, but do not express the gene of interest. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the targeted gene. Such approaches are particularly suited in research and agricultural fields where modifications to embryonic stem cells can be used to generate animal offspring with an inactive targeted gene (e.g., see Thomas & Capecchi 1987 and Thompson 1989, *supra*). However this approach can be routinely adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors that will be apparent to those of skill in the art. The contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety.

Antibodies according to the present invention may be prepared by any of a variety of standard methods using TNFR immunogens of the present invention. Such TNFR immunogens include the TNFR protein shown in Figures 1 and 2 (SEQ ID NO:2 and SEQ ID NO:4, respectively) (which may

or may not include a leader sequence) and polypeptide fragments of TNFR comprising the ligand binding and/or extracellular domains of TNFR.

Polyclonal and monoclonal antibody agonists or antagonists according to the present invention can be raised according to the methods disclosed herein and and/or known in the art, such as, for example, those methods described in Tartaglia and Goeddel, *J. Biol. Chem.* 267(7):4304-4307(1992); Tartaglia *et al.*, *Cell* 73:213-216 (1993), and International application publication number WO 94/09137 (the contents of each of these three applications are herein incorporated by reference in their entireties), and are preferably specific to polypeptides of the invention having the amino acid sequence of SEQ ID NO:2 and/or SEQ ID NO:4. Antibodies according to the present invention may be prepared by any of a variety of methods described herein, and known in the art.

Further antagonist according to the present invention include soluble forms of TNFR, e.g., TNFR fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize TNFR mediated signaling by competing with the cell surface TNFR for binding to TNF-family ligands and/or antagonize TNFR mediated inhibition of apoptosis by, for example, disrupting the ability of TNFR to multimerize and/or to bind to and thereby neutralize apoptosis inducing ligands, such as, for example, Fas ligand and AIM-II.. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of reducing TNFR-mediated inhibition of tumor necrosis induced by TNF-family ligands. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med.* 182:1395-1401 (1995)).

Proteins and other compounds which bind the extracellular domains are also candidate agonist and antagonist according to the present invention. Such

binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature* 340:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (Gyuris, J. et al., *Cell* 75:791-803 (1993); Zervos, A.S. et al., *Cell* 72:223-232 (1993)).

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor family and inducing the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, the TNFR-6 $\alpha$  & -6 $\beta$  ligands, TNF- $\alpha$ , lymphotoxin- $\alpha$  (LT- $\alpha$ , also known as TNF- $\beta$ ), LT- $\beta$ , FasL, CD40, CD27, CD30, 4-IBB, OX40, TRAIL, AIM-II, and nerve growth factor (NGF).

#### ***Formulation and Administration***

The TNFR polypeptide composition will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with TNFR-6 $\alpha$  or -6 $\beta$  polypeptide alone), the site of delivery of the TNFR polypeptide composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" of TNFR polypeptide for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of TNFR polypeptide administered parenterally per dose will be in the range of about 1  $\mu$ g/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the TNFR

polypeptide is typically administered at a dose rate of about 1  $\mu\text{g}/\text{kg}/\text{hour}$  to about 50  $\mu\text{g}/\text{kg}/\text{hour}$ , either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

Effective dosages of the compositions of the present invention to be administered may be determined through procedures well known to those in the art which address such parameters as biological half-life, bioavailability, and toxicity. Such determination is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

Bioexposure of an organism to TNFR-6 $\alpha$  or -6 $\beta$  polypeptide during therapy may also play an important role in determining a therapeutically and/or pharmacologically effective dosing regime. Variations of dosing such as repeated administrations of a relatively low dose of TNFR-6 $\alpha$  or -6 $\beta$  polypeptide for a relatively long period of time may have an effect which is therapeutically and/or pharmacologically distinguishable from that achieved with repeated administrations of a relatively high dose of TNFR-6 $\alpha$  or -6 $\beta$  polypeptide for a relatively short period of time.

Using the equivalent surface area dosage conversion factors supplied by Freireich, E. J., et al. (*Cancer Chemotherapy Reports* 50(4):219-44 (1966)), one of ordinary skill in the art is able to conveniently convert data obtained from the use of TNFR-6 $\alpha$  or -6 $\beta$  polypeptide in a given experimental system into an accurate estimation of a pharmaceutically effective amount of TNFR-6 $\alpha$  or -6 $\beta$  polypeptide to be administered per dose in another experimental system. Experimental data obtained through the administration of TNFR6-Fc in mice (see, for instance, Example 21) may converted through the conversion factors supplied by Freireich, et al., to accurate estimates of pharmaceutically

effective doses of TNFR-6 in rat, monkey, dog, and human. The following conversion table (Table IV) is a summary of the data provided by Freireich, et al. Table IV gives approximate factors for converting doses expressed in terms of mg/kg from one species to an equivalent surface area dose expressed as mg/kg in another species tabulated.

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**Table IV. Equivalent Surface Area Dosage Conversion Factors.**

		<i>--TO--</i>				
		Mouse	Rat	Monkey	Dog	Human
<i>--FROM--</i>		(20g)	(150g)	(3.5kg)	(8kg)	(60kg)
10	Mouse	1	1/2	1/4	1/6	1/12
	Rat	2	1	1/2	1/4	1/7
15	Monkey	4	2	1	3/5	1/3
	Dog	6	4	5/3	1	1/2
	Human	12	7	3	2	1

Thus, for example, using the conversion factors provided in Table IV, a dose of 50 mg/kg in the mouse converts to an appropriate dose of 12.5 mg/kg in the monkey because  $(50 \text{ mg/kg}) \times (1/4) = 12.5 \text{ mg/kg}$ . As an additional example, doses of 0.02, 0.08, 0.8, 2, and 8 mg/kg in the mouse equate to effect doses of 1.667 micrograms/kg, 6.67 micrograms/kg, 66.7 micrograms/kg, 166.7 micrograms/kg, and 0.667 mg/kg, respectively, in the human.

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TNFR-6 alpha and/or TNFR-6 beta polypeptides of the invention may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle accelerators, gelfoam sponge depots, other commercially available depot materials, osmotic pumps, oral or suppositorial solid pharmaceutical formulations, decanting or topical applications during surgery, aerosol delivery. Such methods are known in the art. TNFR-6 alpha and/or TNFR-6 polypeptides of the invention may

be administered as part of a pharmaceutical composition, described in more detail below. Methods of delivering TNFR-6 alpha and/or TNFR-6 beta polynucleotides of the invention are known in the art and described in more detail herein.

5 Pharmaceutical compositions containing the TNFR of the invention may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), bucally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

10 The TNFR polypeptide is also suitably administered by sustained-release systems. Suitable examples of sustained-release compositions include suitable polymeric materials (such as, for example, semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules), suitable hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, and sparingly soluble derivatives (such as, for example, a sparingly soluble salt).

15 Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman, U. et al., *Biopolymers* 22:547-556 (1983)), poly (2-hydroxyethyl methacrylate) (R. Langer et al., *J. Biomed. Mater. Res.* 15:167-277 (1981), and R. Langer, *Chem. Tech.* 12:98-105 (1982)), ethylene

vinyl acetate (R. Langer et al., Id.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988).

Sustained-release compositions also include liposomally entrapped compositions of the invention (see generally, Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 317 -327 and 353-365 (1989)). Liposomes containing TNFR polypeptides may be prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. (USA) 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci. (USA) 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal TNFR polypeptide therapy.

In yet an additional embodiment, the compositions of the invention are delivered by way of a pump (see Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

For parenteral administration, in one embodiment, the TNFR polypeptide is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.

Generally, the formulations are prepared by contacting the TNFR polypeptide uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, manose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

The TNFR polypeptide is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of TNFR polypeptide salts.

TNFR polypeptides to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutic TNFR polypeptide

compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

TNFR polypeptides ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous TNFR polypeptide solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized TNFR polypeptide using bacteriostatic Water-for-Injection.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides of the present invention may be employed in conjunction with other therapeutic compounds.

The compositions of the invention may be administered alone or in combination with other therapeutic agents, including but not limited to, chemotherapeutic agents, anti-opportunistic infection agents, antivirals, antibiotics, steroidal and non-steroidal anti-inflammatories, immunosuppressants, conventional immunotherapeutic agents and cytokines. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination"

further includes the separate administration of one of the compounds or agents given first, followed by the second.

In one embodiment, the compositions of the invention are administered in combination with other members of the TNF family. TNF, TNF-related or TNF-like molecules that may be administered with the compositions of the invention include, but are not limited to, soluble forms of TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, CD27L, CD30L, CD40L, 4-IBBL, DcR3, OX40L, TNF-gamma International application publication number WO 96/14328), AIM-I (International application publication number WO 97/33899), AIM-II (International application publication number WO 97/34911), APRIL (*J. Exp. Med.* 188(6):1185-1190), endokine-alpha (International Publication No. WO 98/07880), OPG, and neutrokinin-alpha (International application publication number WO 98/18921), TWEAK, OX40, and nerve growth factor (NGF), and soluble forms of Fas, CD30, CD27, CD40 and 4-IBB, TR2 (International application publication number WO 96/34095), DR3 (International Publication No. WO 97/33904), DR4 (International application publication number WO 98/32856), TR5 (International application publication number WO 98/30693), TR7 (International application publication number WO 98/41629), TRANK, TR9 (International application publication number WO 98/56892), TR10 (International application publication number WO 98/54202), 312C2 (International application publication number WO 98/06842), and TR12.

Conventional nonspecific immunosuppressive agents, that may be administered in combination with the compositions of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methylprednisolone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents that act by suppressing the function of responding T cells.

In specific embodiments, compositions of the invention are administered in combination with immunosuppressants.

Immunosuppressants preparations that may be administered with the compositions of the invention include, but are not limited to,

5           ORTHOCLOONE™ (OKT3), SANDIMMUNE™/NEORAL™/SANGDYA™ (cyclosporin), PROGRAF™ (tacrolimus), CELLCEPT™ (mycophenolate), Azathioprine, glucorticosteroids, and RAPAMUNE™ (sirolimus). In a specific embodiment, immunosuppressants may be used to prevent rejection of organ or bone marrow transplantation.

10          In certain embodiments, compositions of the invention are administered in combination with antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors. Nucleoside reverse transcriptase inhibitors that may be administered in combination with the compositions of the invention, include, but are not limited to, RETROVIR™ (zidovudine/AZT), VIDEX™ (didanosine/ddI), HIVID™ (zalcitabine/ddC), ZERIT™ (stavudine/d4T), EPIVIR™ (lamivudine/3TC), and COMBIVIR™ (zidovudine/lamivudine).

15          Non-nucleoside reverse transcriptase inhibitors that may be administered in combination with the compositions of the invention, include, but are not limited to, VIRAMUNE™ (nevirapine), REScriptor™ (delavirdine), and SUSTIVA™ (efavirenz). Protease inhibitors that may be administered in combination with the compositions of the invention, include, but are not limited to, CRIXIVAN™ (indinavir), NORVIR™ (ritonavir), INVIRASE™ (saquinavir), and VIRACEPT™ (nelfinavir). In a specific embodiment, 20        antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors may be

used in any combination with compositions of the invention to treat AIDS and/or to prevent or treat HIV infection.

In other embodiments, compositions of the invention may be administered in combination with anti-opportunistic infection agents. Anti-opportunistic agents that may be administered in combination with the compositions of the invention, include, but are not limited to,  
5 TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™,  
PENTAMIDINE™, ATOVAQUONE™, ISONIAZID™, RIFAMPIN™,  
PYRAZINAMIDE™, ETHAMBUTOL™, RIFABUTIN™,  
10 CLARITHROMYCIN™, AZITHROMYCIN™, GANCICLOVIR™,  
FOSCARNET™, CIDOFOVIR™, FLUCONAZOLE™,  
ITRACONAZOLE™, KETOCONAZOLE™, ACYCLOVIR™,  
FAMCICOLVIR™, PYRIMETHAMINE™, LEUCOVORIN™,  
15 NEUPOGEN™ (filgrastim/G-CSF), and LEUKINE™ (sargramostim/GM-CSF). In a specific embodiment, compositions of the invention are used in any combination with TRIMETHOPRIM-SULFAMETHOXAZOLE™,  
DAPSONE™, PENTAMIDINE™, and/or ATOVAQUONE™ to  
prophylactically treat or prevent an opportunistic *Pneumocystis carinii*  
pneumonia infection. In another specific embodiment, compositions of the  
20 invention are used in any combination with ISONIAZID™, RIFAMPIN™,  
PYRAZINAMIDE™, and/or ETHAMBUTOL™ to prophylactically treat or  
prevent an opportunistic *Mycobacterium avium* complex infection. In another  
specific embodiment, compositions of the invention are used in any  
combination with RIFABUTIN™, CLARITHROMYCIN™, and/or  
25 AZITHROMYCIN™ to prophylactically treat or prevent an opportunistic  
*Mycobacterium tuberculosis* infection. In another specific embodiment,  
compositions of the invention are used in any combination with

GANCICLOVIR™, FOSCARNET™, and/or CIDOFOVIR™ to prophylactically treat or prevent an opportunistic cytomegalovirus infection. In another specific embodiment, compositions of the invention are used in any combination with FLUCONAZOLE™, ITRACONAZOLE™, and/or KETOCONAZOLE™ to prophylactically treat or prevent an opportunistic fungal infection. In another specific embodiment, compositions of the invention are used in any combination with ACYCLOVIR™ and/or FAMCICOLVIR™ to prophylactically treat or prevent an opportunistic herpes simplex virus type I and/or type II infection. In another specific embodiment, compositions of the invention are used in any combination with PYRIMETHAMINE™ and/or LEUCOVORIN™ to prophylactically treat or prevent an opportunistic *Toxoplasma gondii* infection. In another specific embodiment, compositions of the invention are used in any combination with LEUCOVORIN™ and/or NEUPOGEN™ to prophylactically treat or prevent an opportunistic bacterial infection.

In a further embodiment, the compositions of the invention are administered in combination with an antiviral agent. Antiviral agents that may be administered with the compositions of the invention include, but are not limited to, acyclovir, ribavirin, amantadine, and remantidine

In a further embodiment, the compositions of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the compositions of the invention include, but are not limited to, amoxicillin, aminoglycosides, beta-lactam (glycopeptide), beta-lactamases, Clindamycin, chloramphenicol, cephalosporins, ciprofloxacin, ciprofloxacin, erythromycin, fluoroquinolones, macrolides, metronidazole, penicillins, quinolones, rifampin, streptomycin, sulfonamide, tetracyclines, trimethoprim, trimethoprim-sulfamthoxazole, and vancomycin.

In an additional embodiment, the compositions of the invention are administered alone or in combination with an anti-inflammatory agent. Anti-inflammatory agents that may be administered with the compositions of the invention include, but are not limited to, glucocorticoids and the nonsteroidal anti-inflammatories, aminoarylcarboxylic acid derivatives, arylacetic acid derivatives, arylbutyric acid derivatives, arylcarboxylic acids, arylpropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, bendazac, benzydamine, bucolome, difenpiramide, ditazol, emorfazole, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

In another embodiment, compositions of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents that may be administered with the compositions of the invention include, but are not limited to, antibiotic derivatives (e.g., doxorubicin, bleomycin, daunorubicin, and dactinomycin); antiestrogens (e.g., tamoxifen); antimetabolites (e.g., fluorouracil, 5-FU, methotrexate, floxuridine, interferon alpha-2b, glutamic acid, plicamycin, mercaptopurine, and 6-thioguanine); cytotoxic agents (e.g., carmustine, BCNU, lomustine, CCNU, cytosine arabinoside, cyclophosphamide, estramustine, hydroxyurea, procarbazine, mitomycin, busulfan, cis-platin, and vincristine sulfate); hormones (e.g., medroxyprogesterone, estramustine phosphate sodium, ethinyl estradiol, estradiol, megestrol acetate, methyltestosterone, diethylstilbestrol diphosphate, chlorotrianisene, and testolactone); nitrogen mustard derivatives (e.g., mephalen, chorambucil, mechlorethamine (nitrogen mustard) and thiotapec); steroids and combinations (e.g., bethamethasone sodium phosphate); and others (e.g., dicarbazine, asparaginase, mitotane, vincristine sulfate, vinblastine sulfate, and etoposide).

In a specific embodiment, compositions of the invention are administered in combination with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or any combination of the components of CHOP.

5 In another embodiment, compositions of the invention are administered in combination with Rituximab. In a further embodiment, compositions of the invention are administered with Rituxmab and CHOP, or Rituxmab and any combination of the components of CHOP.

10 In an additional embodiment, the compositions of the invention are administered in combination with cytokines. Cytokines that may be administered with the compositions of the invention include, but are not limited to, GM-CSF, G-CSF, IL2, IL3, IL4, IL5, IL6, IL7, IL10, IL12, IL13, IL15, anti-CD40, CD40L, IFN-alpha, IFN-beta, IFN-gamma, TNF-alpha, and TNF-beta. In another embodiment, compositions of the invention may be administered with any interleukin, including, but not limited to, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, and IL-21. In a preferred embodiment, the compositions of the invention are administered in combination with TNF-alpha. In another preferred embodiment, the compositions of the invention are administered in combination with IFN-alpha.

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25 In an additional embodiment, the compositions of the invention are administered in combination with angiogenic proteins. Angiogenic proteins that may be administered with the compositions of the invention include, but are not limited to, Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816; Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110; Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European Patent Number EP-282317; Placental Growth Factor (PIGF), as disclosed in International Publication Number WO 92/06194; Placental Growth Factor-2

(PIGF-2), as disclosed in Hauser et al., Gorwth Factors, 4:259-268 (1993); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649; Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477; Vascular  
5 Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515; Vascular Endothelial Growth Factor B-186 (VEGF-B186), as disclosed in International Publication Number WO 96/26736; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/02543; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832; and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601. The above mentioned references are incorporated herein by reference herein.

In an additional embodiment, the compositions of the invention are administered in combination with Fibroblast Growth Factors. Fibroblast Growth Factors that may be administered with the compositions of the invention include, but are not limited to, FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, and FGF-15.

In additional embodiments, the compositions of the invention are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

#### *Chromosome Assays*

The nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence

data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

5           In certain preferred embodiments in this regard, the cDNAs herein disclosed are used to clone genomic DNA of a TNFR protein gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA then is used for *in situ* chromosome mapping using well known techniques for this purpose.

10           In addition, in some cases, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. 15           Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with probes from the cDNA as short as 50 or 60 bp. For a review of this technique, see Verma *et al.*, *Human Chromosomes: A Manual Of Basic Techniques*, Pergamon Press, New York (1988).

20           Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance In Man*, available on-line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

### ***Examples***

#### ***Example 1: Expression and Purification of TNFR-6 alpha and TNFR-6 beta in E. coli***

The bacterial expression vector pQE60 is used for bacterial expression in this example (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Ampr") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin sold by QIAGEN, Inc., *supra*, and suitable single restriction enzyme cleavage sites. These elements are arranged such that a DNA fragment encoding a polypeptide may be inserted in such a way as to produce that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the carboxyl terminus of that polypeptide. However, in this example, the polypeptide coding sequence is inserted such that translation of the six His codons is prevented and, therefore, the polypeptide is produced with no 6 X His tag.

The DNA sequences encoding the desired portions of TNFR-6 alpha and TNFR-6 beta proteins comprising the mature forms of the TNFR-6 alpha and TNFR-6 beta amino acid sequences are amplified from the deposited

cDNA clones using PCR oligonucleotide primers which anneal to the amino terminal sequences of the desired portions of the TNFR-6 $\alpha$  or -6 $\beta$  proteins and to sequences in the deposited constructs 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning the mature form of the TNFR-6 $\alpha$  protein, the 5' primer has the sequence 5' CGCCCATGGCAGAACACCCACCTAC 3' (SEQ ID NO:19) containing the underlined NcoI restriction site. One of ordinary skill in the art would appreciate, of course, that the point in the protein coding sequence where the 5' primer begins may be varied to amplify a desired portion of the complete protein shorter or longer than the mature form. The 3' primer has the sequence 5' CGCAAGCTTCTTTCA GTGCAAGTG 3' (SEQ ID NO:20) containing the underlined HindIII restriction site. For cloning the mature form of the TNFR-6 $\beta$  protein, the 5' primer has the sequence of SEQ ID NO:19 above, and the 3' primer has the sequence 5' CGCAAGCTTCTCCTCAGCTCCTGCAGTG 3' (SEQ ID NO:21) containing the underlined HindIII restriction site.

The amplified TNFR-6 alpha and TNFR-6 beta DNA fragments and the vector pQE60 are digested with NcoI and HindIII and the digested DNAs are then ligated together. Insertion of the TNFR-6 alpha and TNFR-6 beta DNA into the restricted pQE60 vector places the TNFR-6 alpha and TNFR-6 beta protein coding region including its associated stop codon downstream from the IPTG-inducible promoter and in-frame with an initiating AUG. The associated stop codon prevents translation of the six histidine codons downstream of the insertion point.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described in Sambrook *et al.*, *Molecular*

Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kanr"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing TNFR-6 $\alpha$  or -6 $\beta$  protein, is available commercially from QIAGEN, Inc., *supra*. Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100  $\mu$ g/ml) and kanamycin (25  $\mu$ g/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD600") of between 0.4 and 0.6. isopropyl- $\beta$ -D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the *lac* repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

To purify the TNFR-6 alpha and TNFR-6 beta polypeptide, the cells are then stirred for 3-4 hours at 4° C in 6M guanidine-HCl, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the TNFR-6 alpha and TNFR-6 beta is dialyzed against 50 mM Na-acetate buffer pH 6, supplemented with 200 mM NaCl. Alternatively, the protein can be successfully refolded by dialyzing it against 500 mM NaCl, 20% glycerol, 25 mM Tris/HCl pH 7.4, containing protease inhibitors. After renaturation the protein can be purified by ion exchange, hydrophobic interaction and size

exclusion chromatography. Alternatively, an affinity chromatography step such as an antibody column can be used to obtain pure TNFR-6 alpha and TNFR-6 beta protein. The purified protein is stored at 4° C or frozen at -80° C.

5           The following alternative method may be used to purify TNFR-6 $\alpha$  or -6 $\beta$  expressed in *E coli* when it is present in the form of inclusion bodies. Unless otherwise specified, all of the following steps are conducted at 4-10°C.

10           Upon completion of the production phase of the *E. coli* fermentation, the cell culture is cooled to 4-10°C and the cells are harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution containing 100 mM Tris, 50 mM EDTA, pH 7.4. The cells are dispersed to a homogeneous suspension using a high shear mixer.

15           The cells were then lysed by passing the solution through a microfluidizer (Microfluidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by centrifugation at 7000 xg for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

20           The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GnHCl) for 2-4 hours. After 7000 xg centrifugation for 15 min., the pellet is discarded and the TNFR-6 $\alpha$  or -6 $\beta$  polypeptide-containing supernatant is incubated at 4°C overnight to allow further GnHCl extraction.

25           Following high speed centrifugation (30,000 xg) to remove insoluble particles, the GnHCl solubilized protein is refolded by quickly mixing the

GnHCl extract with 20 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4°C without mixing for 12 hours prior to further purification steps.

5 To clarify the refolded TNF receptor polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16 µm membrane filter with appropriate surface area (e.g., Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perseptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a stepwise manner. The absorbance at 280 nm of the effluent is continuously monitored. Fractions are collected and further analyzed by SDS-PAGE.

10 Fractions containing the TNF receptor polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perseptive Biosystems) and weak anion (Poros CM-20, Perseptive Biosystems) exchange resins. The columns are equilibrated with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under constant  $A_{280}$  monitoring of the effluent. Fractions containing the TNFR-6 $\alpha$  or -6 $\beta$  polypeptide (determined, for instance, by 16% SDS-PAGE) are then pooled.

15 The resultant TNF receptor polypeptide exhibits greater than 95% purity after the above refolding and purification steps. No major contaminant

bands are observed from Commassie blue stained 16% SDS-PAGE gel when 5 µg of purified protein is loaded. The purified protein is also tested for endotoxin/LPS contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

5       ***Example 2: Cloning and Expression of TNFR-6 alpha and TNFR-6 beta proteins in a Baculovirus Expression System***

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding complete protein, including its naturally associated secretory signal (leader) sequence, into a baculovirus to express the mature TNFR-6 $\alpha$  or -6 $\beta$  protein, using standard methods as described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites such as BamHI, Xba I and Asp718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak Drosophila promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate a viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal

peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow *et al.*, *Virology* 170:31-39 (1989).

The cDNA sequence encoding the full length TNFR-6 $\alpha$  or -6 $\beta$  protein in a deposited clone, including the AUG initiation codon and the naturally associated leader sequence shown in SEQ ID NO:2 or 4 is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene.

5 The 5' primer for TNFR-6 alpha and TNFR-6 beta has the sequence

5' CGCGGATCCGCCATCATGAGGGCGTGGAGGGGCCAG 3' (SEQ

10 ID NO:22) containing the underlined BamHI restriction enzyme site. All of the previously described primers encode an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.*

15 196:947-950 (1987). The 3' primer for TNFR-6 $\alpha$  has the sequence

5' CGCGGTACCCTCTTCAGTGCAAGTG 3' (SEQ ID NO:23)

containing the underlined Asp718 restriction site. The 3' primer for TNFR-6 $\beta$  has the sequence 5' CGCGGTACCCTCCTCAGCTCCTGCAGTG 3' (SEQ 15 ID NO:24) containing the underlined Asp718 restriction site.

20 The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with the appropriate restriction enzyme for each of the primers used, as specified above, and again is purified on a 1% agarose gel.

25 The plasmid is digested with the same restriction enzymes and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.).

The fragment and dephosphorylated plasmid are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1

Blue (Statagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human TNF receptor gene by digesting DNA from individual colonies using the enzymes used immediately above and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pA2-TNFR-6 $\alpha$  or pA2TNFR-6 $\beta$  (collectively pA2-TNFR).

Five  $\mu$ g of the plasmid pA2-TNFR is co-transfected with 1.0  $\mu$ g of a commercially available linearized baculovirus DNA ("BaculoGold<sup>TM</sup> baculovirus DNA", Pharmingen, San Diego, CA), using the lipofection method described by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84: 7413-7417 (1987). One  $\mu$ g of BaculoGold<sup>TM</sup> virus DNA and 5  $\mu$ g of the plasmid pA2-TNFR are mixed in a sterile well of a microtiter plate containing 50  $\mu$ l of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10  $\mu$ l Lipofectin plus 90  $\mu$ l Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is then incubated for 5 hours at 27° C. The transfection solution is then removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. Cultivation is then continued at 27° C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, *supra*. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology

distributed by Life Technologies Inc., Gaithersburg, page 9-10). After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 µl of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4° C.

To verify the expression of the TNF receptor gene Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus at a multiplicity of infection ("MOI") of about 2. If radiolabeled proteins are desired, 6 hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). After 42 hours, 5 µCi of  $^{35}\text{S}$ -methionine and 5 µCi  $^{35}\text{S}$ -cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled).

Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the mature form of the TNF receptor protein.

***Example 3: Cloning and Expression of TNFR-6 alpha and TNFR-6 beta in Mammalian Cells***

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers,

Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLV, HIVI and the early promoter of the 5 cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 10 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 15 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and 15 isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy *et al.*, *Biochem J.* 227:277-279 (1991); Bebbington *et al.*, *Bio/Technology* 10:169-175 (1992)). Using these markers, the mammalian 20 cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a 25 chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology*, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

**Example 3(a): Cloning and Expression in COS Cells**

The expression plasmid, pTNFR- $\alpha$ -HA and -6 $\beta$ -HA, is made by cloning a portion of the cDNA encoding the mature form of the TNF receptor protein into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson *et al.*, *Cell* 37: 767 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the complete TNF receptor polypeptide is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The TNF receptor cDNA of a deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of a TNF receptor in *E. coli*. Suitable primers can easily be designed by those of ordinary skill in the art.

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with XbaI and EcoRI and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the fragment encoding the TNFR- $\alpha$  and -6 $\beta$  polypeptides.

For expression of recombinant TNFR- $\alpha$  and -6 $\beta$ , COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of TNFR by the vector.

Expression of the pTNFR- $\alpha$ -HA and -6 $\beta$ -HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow *et al.*, *Antibodies: A Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To

this end, two days after transfection, the cells are labeled by incubation in media containing  $^{35}\text{S}$ -cysteine for 8 hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 5 50 mM TRIS, pH 7.5, as described by Wilson *et al.* cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

10 ***Example 3(b): Cloning and Expression in CHO Cells***

The vector pC4 is used for the expression of TNFR-6 alpha and TNFR-6 beta polypeptides. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., 1978, *J. Biol. Chem.* 253:1357-1370, Hamlin, J. L. and Ma, C. 1990, *Biochem. et Biophys. Acta*, 1097:107-143, Page, M. J. and Sydenham, M. A. 1991, *Biotechnology* 9:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop 20 cell lines carrying more than 1,000 copies of the amplified gene(s).

Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, *et al.*, *Molecular and Cellular Biology*, March 1985:438-447) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al.*, *Cell* 41:521-530 (1985)). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, Xba I, and Asp718. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human  $\beta$ -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the TNF receptor polypeptide in a regulated way in mammalian cells (Gossen, M., & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzymes appropriate for the specific primers used to amplify the TNF receptor of choice as outlined below and then dephosphorylated using calf intestinal phosphates by procedures

known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the TNF receptor polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the desired portion of the gene. The 5' primer for TNFR-6 alpha and TNFR-6 beta containing the underlined BamHI site, has the following sequence:

5' CGCGGATCCGCCATCATGAGGGCGTGGAGGGGCCAG 3' (SEQ ID NO:22). The 3' primer for TNFR-6 $\alpha$  has the sequence

5' CGCGGTACCCTTTCA GTGCAAGTG 3' (SEQ ID NO:23)

containing the underlined Asp718 restriction site. The 3' primer for TNFR-6 $\beta$  has the sequence 5' CGCGGTACCCTCAGCTCAGCTGCAGTG 3' (SEQ ID NO:24) containing the underlined Asp718 restriction site.

The amplified fragment is digested with the endonucleases which will cut at the engineered restriction site(s) and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five  $\mu$ g of the expression plasmid pC4 is cotransfected with 0.5  $\mu$ g of the plasmid pSVneo using lipofectin (Felgner *et al.*, *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks

using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1  $\mu$ M, 2  $\mu$ M, 5  $\mu$ M, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 5 200  $\mu$ M. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

***Example 4: Tissue distribution of TNF receptor mRNA expression***

10 Northern blot analysis is carried out to examine TNFR-6 $\alpha$  or -6 $\beta$  gene expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of a TNF receptor protein (SEQ ID NO:1 or 3) is labeled with  $^{32}$ P using the *rediprime*<sup>TM</sup> DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified 15 using a CHROMA SPIN-100<sup>TM</sup> column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for TNF receptor mRNA.

20 Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with the labeled probe using ExpressHyb<sup>TM</sup> hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70° C overnight, and films developed according to standard 25 procedures.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of

the above teachings and, therefore, are within the scope of the appended claims.

**Example 5: Gene Therapy Using Endogenous TNFR-6 Gene**

Another method of gene therapy according to the present invention involves operably associating the endogenous TNFR (i.e., TNFR-6) sequence with a promoter via homologous recombination as described, for example, in U.S. Patent No. 5,641,670, issued June 24, 1997; International application publication number WO 96/29411, published September 26, 1996; International application publication number WO 94/12650, published August 4, 1994; Koller *et al.*, *Proc. Natl. Acad. Sci. USA* 86:8932-8935 (1989); and Zijlstra *et al.*, *Nature* 342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not expressed in the cells, or is expressed at a lower level than desired.

Polynucleotide constructs are made which contain a promoter and targeting sequences, which are homologous to the 5' non-coding sequence of endogenous TNFR-6, flanking the promoter. The targeting sequence will be sufficiently near the 5' end of TNFR-6 so the promoter will be operably linked to the endogenous sequence upon homologous recombination. The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter.

The amplified promoter and the amplified targeting sequences are digested with the appropriate restriction enzymes and subsequently treated with calf intestinal phosphatase. The digested promoter and digested targeting sequences are added together in the presence of T4 DNA ligase. The resulting

mixture is maintained under conditions appropriate for ligation of the two fragments. The construct is size fractionated on an agarose gel then purified by phenol extraction and ethanol precipitation.

In this Example, the polynucleotide constructs are administered as naked polynucleotides via electroporation. However, the polynucleotide constructs may also be administered with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, precipitating agents, etc. Such methods of delivery are known in the art.

Once the cells are transfected, homologous recombination will take place which results in the promoter being operably linked to the endogenous TNFR-6 sequence. This results in the expression of TNFR-6 in the cell. Expression may be detected by immunological staining, or any other method known in the art.

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in DMEM + 10% fetal calf serum. Exponentially growing or early stationary phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining cells are subjected to centrifugation. The supernatant is aspirated and the pellet is resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains approximately 3X10<sup>6</sup> cells/ml. Electroporation should be performed immediately following resuspension.

Plasmid DNA is prepared according to standard techniques. For example, to construct a plasmid for targeting to the TNFR-6 locus, plasmid pUC18 (MBI Fermentas, Amherst, NY) is digested with HindIII. The CMV promoter is amplified by PCR with an XbaI site on the 5' end and a BamHI

site on the 3' end. Two TNFR-6 non-coding sequences are amplified via PCR: one TNFR-6 non-coding sequence (TNFR-6 fragment 1) is amplified with a HindIII site at the 5' end and an Xba site at the 3' end; the other TNFR-6 non-coding sequence (TNFR-6 fragment 2) is amplified with a BamHI site at the 5'end and a HindIII site at the 3' end. The CMV promoter and TNFR-6 fragments are digested with the appropriate enzymes (CMV promoter - XbaI and BamHI; TNFR-6 fragment 1 - XbaI; TNFR-6 fragment 2 - BamHI) and ligated together. The resulting ligation product is digested with HindIII, and ligated with the HindIII-digested pUC18 plasmid.

10 Plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio- Rad). The final DNA concentration is generally at least 120 µg/ml. 0.5 ml of the cell suspension (containing approximately  $1.5 \times 10^6$  cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad).

15 Capacitance and voltage are set at 960 µF and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

20 Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (DMEM with 15% calf serum) in a 10 cm dish and incubated at 37°C. The following day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hours.

25 The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. The fibroblasts can then be introduced into a patient as described above.

**Example 6: Effect of TNFR in treating graft-versus-host disease in mice**

The invention also encompasses a method for the treatment of refractory /severe acute GVHD in patients comprising administering to the patients (preferably human), TNFR polypeptides or TNFR agonists of the invention.

An analysis of the use of soluble TNFR polypeptides of the invention (e.g., TNFR-6) to treat graft-versus-host disease (GVHD) is performed through the use of a C57BL/6 parent into (BALB/c X C57BL/6) F1 mouse model. This parent into F1 mouse model is a well-characterized and reproducible animal model of GVHD in bone marrow transplant patients, which is well known to one of ordinary skill in the art (see, e.g., Gleichemann et al, *Immunol Today* 5:324, 1984, which is herein incorporated by reference in its entirety). Soluble TNFR is expected to bind to FasL and inhibit FasL-mediated apoptosis, which plays a critical pathogenic role in the hepatic, cutaneous and lymphoid organ damage observed in this animal model of GVHD (Baker et al, *J. Exp. Med.* 183:2645, (1996); Charles et al, *J. Immunol.* 157:5387, (1996); and Hattori et al, *Blood* 91:4051, (1998), each of which is herein incorporated by reference in its entirety).

Initiation of the GVHD condition is induced by the intravenous injection of ~1-3 x 10<sup>8</sup> spleen cells from C57BL/6 mice into (BALB/c X C57BL/6) F1 mice (both are available from Jackson Lab, Bar Harbor, Maine). Groups of 6 to 8 mice receive either 0.1 to 5.0 mg/kg of TNFR or human IgG isotype control intraperitoneally or intradermally on every other day following the injection of spleen cells. The effect of TNFR on liver enzyme release in the sera, an indicator of liver damage, is analyzed twice per week for at least 3 weeks. When there is a significant amount of liver enzymes being detected in human IgG-treated mice, the animals are sacrificed for histological evaluation of

the relative degree of tissue damage in the liver, spleen, skin and intestine, and for the therapeutic effect TNFR has elicited on these organs.

The ability of TNFR to ameliorate systems associated with refractory/severe acute GVHD is indicated by a reduction of liver enzyme release in the sera, tissue damage and/or reduced cachexia, loss of body weight and/or lethality when compared to the control.

Finally, TNFR- and human IgG-treated animals undergo a clinical evaluation every other day to assess cachexia, body weight and lethality.

TNFR in combination therapy with TNF- $\alpha$  inhibitors may also be examined in this GVHD murine model.

***Example 7: TNFR-6 $\alpha$  (DcR3) suppresses AIM-II-mediated apoptosis***

**Background**

The members of the tumor necrosis factor (TNF) family are involved in regulating diverse biological activities such as regulation of cell proliferation, differentiation, cell survival, cell death, cytokine production, lymphocyte co-stimulation, immunoglobulin secretion, and isotype switching (Armitage, R., *Curr. Opin. Immunol.* 6, 407-413 (1994); Tewari, M. et al., *Curr. Opin. Genet. Dev.* 6, 39-44 (1996)). Receptors in this family share a common structural motif in their extracellular domains consisting of multiple cysteine-rich repeats of approximately 30 to 40 amino acids (Gruss, H.-J., et al., *Blood* 85, 3378-3404 (1995)). While TNFR1, CD95/Fas/APO-1,

DR3/TRAMP/APO-3, DR4/TRAIL-R1/APO-2, DR5/TRAIL-R2, and DR6 receptors contain a conserved intracellular motif of 30 – 40 amino acids called death domain, associated with the activation of apoptotic signaling pathways, other members which contain a low sequence identity in the intracellular domains, stimulate the transcription factors NF- $\kappa$ B and AP-1 (Armitage, R.,

*Curr. Opin. Immunol.* 6, 407-413 (1994); Tewari, M. *et al.*, *Curr. Opin. Genet. Dev.* 6, 39-44 (1996); Gruss, H.-J. *et al.*, *Blood* 85, 3378-3404 (1995)).

Most TNF receptors contain functional cytoplasmic domain and they include TNFR1 (Loetscher, H *et al.*, *Cell* 61, 351-356 (1990); Schall, T. J., *et al.*, *Cell* 61, 361-370 (1990)), TNFR2 (Smith, C. A., *et al.*, *Science* 248, 1019-1023 (1990)), lymphotoxin  $\beta$  receptor (LT $\beta$ R) (Baens, M., *et al.*, *Genomics* 16, 214-218 (1993)), 4-1BB (Kwon, B. S., *et al.*, *Proc. Natl. Acad. Sci. USA* 86, 1963-1967 (1989)), HVEM/TR2/ATAR (Kwon, B. S., *et al.*, *J. Biol. Chem.* 272, 14272-14276 (1997); Montgomery, R. I., *et al.*, *Cell* 87, 427-436 (1996); Hsu, H., *et al.*, *J. Biol. Chem.* 272, 13471-13474 (1997)), NGFR (Johnson, D., *et al.*, *Cell* 47, 545-554 (1986)), CD27 (Van Lier, R. A., *et al.*, *J. Immunol.* 139, 1589-1596 (1987)), CD30 (Durkorp, H., *et al.*, *Cell* 68, 421-427 (1992)), CD40 (Banchereau, J., *et al.*, *Cell* 68, 421-427 (1994)), OX40 (Mallett, S., *et al.*, *EMBO J.* 9, 1063-1068 (1990)), Fas (Itoh, N., *et al.*, *Cell* 66, 233-243 (1991)), DR3/TRAMP (Chinnaiyan, A. M., *et al.*, *Science* 274, 990-992 (1996)), DR4/TRAIL-R1 (Pan, G., *et al.*, *Science* 276, 111-113 (1996)), DR5/TRAIL-R2 (Pan, G., *et al.*, *Science* 277, 815-818) (1997), and RANK (Anderson, D. *et al.*, *Nature* 390, 175-179 (1997)). Some members of the TNFR superfamily do not have cytoplasmic domains and are secreted, such as osteoprotegerin (OPG) (Simmonet, *et al.*, *Cell* 89, 309-319 (1997)), or linked to the membrane through a glycophospholipid tail, such as TRID/DcR1/TRAIL-R3 (Degli-Esposti, M. A., *et al.*, *J. Exp. Med.* 186, 1165-1170 (1997); Sheridan, J. P., *et al.*, *Science* 277, 818-821 (1997)). Viral open reading frames encoding soluble TNFRs have also been identified, such as SFV-T2 (Smith, C. A., *et al.*, *Science* 248, 1019-1023 (1990)), Va53 (Howad, S. T., *et al.*, *Virology* 180, 633-647 (1991)), G4RG (Hu, F. Q., *et al.*, *Virology* 204, 343-356 (1994)), and crmB (Gruss, H.-J., *et al.*, *Blood* 85, 3378-3404 (1995)).

By searching an expressed sequence tag (EST) database, a new member of the TNFR superfamily was identified, named TNFR-6 $\alpha$ , and was characterized as a soluble cognate ligand for AIM-II and FasL/CD95L. AIM-II and FasL mediate the apoptosis, which is the most common physiological form of cell death and occurs during embryonic development, tissue remodeling, immune regulation and tumor regression.

AIM-II is highly induced in activated T lymphocytes and macrophages. AIM-II was characterized as a cellular ligand for HVEM/TR2 and LT $\beta$ R (Mauri, D. N., *et al.*, *Immunity* 8, 21-30 (1998)). HVEM/TR2 is a receptor for herpes simplex virus type 1 (HSV-1) entry into human T lymphoblasts. Soluble form of HVEM/TR2-Fc and antibodies to HVEM/TR2 were shown to inhibit a mixed lymphocyte reaction, suggesting a role for this receptor or its ligand in T lymphocyte proliferation (Kwon, B. *et al.*, *J. Biol. Chem.* 272, 14272-14276 (1997); Mauri, D. N., *et al.*, *Immunity* , 21-30 (1998); Harrop, J. A., *et al.*, *J. Immunol.* 161, 1786-1794 (1998)). The level of LT $\beta$ R expression is prominent on epithelial cells but is absent in T and B lymphocytes. Signaling via LT $\beta$ R triggers cell death in some adenocarcinomas (Browning, J. L., *et al.*, *J. Exp. Med.* 183, 867-878 (1996)). AIM-II produced by activated lymphocytes could evoke immune modulation from hematopoietic cells expressing only HVEM/TR2, and induce apoptosis of tumor cells, which express both LT $\beta$ R and HVEM/TR2 receptors (Zhai, Y., *et al.*, *J. Clin. Invest.* 102, 1142-1151 (1998); Harrop, J. A., *et al.*, *J. Biol. Chem.* 273, 27548-27556 (1998)).

FasL is one of the major effectors of cytotoxic T lymphocytes and natural killer cells. It is also involved in the establishment of peripheral tolerance, in the activation-induced cell death of lymphocytes. Moreover, expression of FasL in nonlymphoid and tumor cells contributes to the maintenance of immune privilege of tissues by preventing the infiltration of

Fas-sensitive lymphocytes (Nagata, S., *Cell* 88, 355-365 (1997)). FasL is also processed and shed from the surface of human cell (Schneider, P., *et al.*, *J. Exp. Med.* 187, 1205-1213 (1998)).

Here, we demonstrate that TNFR-6 $\alpha$ , a new member of the TNFR superfamily binds AIM-II and FasL. Therefore TNFR-6 $\alpha$ , may act as an inhibitor in AIM-II -induced tumor cell death by blocking AIM-II interaction with its receptors.

## Materials and Methods

### *Identification and cloning of new members of the TNFR superfamily.*

An EST cDNA database, obtained from more than 600 different cDNA libraries, was screened for sequence homology with the cysteine-rich motif of the TNFR superfamily, using the blastn and tblastn algorithms. Three EST clones containing an identical open reading frame whose amino acid sequence showed significant homology to TNFR-II were identified from cDNA libraries of human normal prostate and pancreas tumor. A full-length TNFR-6 alpha cDNA clone encoding an intact N-terminal signal peptide was obtained from a human normal prostate library.

### *RT-PCR analysis.*

For RT-PCR analysis, total RNA was isolated using Trizol (GIBCO) from various human cell lines before and after stimulation with PMA/Ionomycin or LPS. RNA was converted to cDNA by reverse transcription and amplified for 35 cycles by PCR. Primers used for amplification of the TNFR-6 alpha fragment are according to the sequence of TNFR-6 alpha.  $\beta$ -actin was used as an internal control for RNA integrity. PCR products were run on 2% agarose gel, stained with ethidium bromide and visualized by UV illumination.

### *Recombinant protein production and purification.*

The recombinant TNFR-6 alpha protein was produced with hexa-histidine at the C-terminus. TNFR-6 alpha-(His) encoding the entire TNFR-6 alpha protein was amplified by PCR. For correctly oriented cloning, a HindIII site on the 5' end of the forward primer (5'-AGACCCAAGCTTC  
5 CTGCTCCA GCAAGGACCATG-3':SEQ ID NO:25) and a BamHI site on the 5' end of the reverse primer (5'-  
AGACGGGATCCTTAGTGGTGGTGGTGGTGCAC  
AGGGAGGAAGCGCTC-3':SEQ ID NO:26) were created. The amplified fragment was cut with HindIII/BamHI and cloned into mammalian expression vector, pCEP4 (Invitrogen). The TNFR-6 alpha-(His)/pCEP4 plasmid was stably transfected into HEK 293 EBNA cells to generate recombinant TNFR-6 alpha-(His). Serum free culture media from cells transfected TNFR-6 alpha-(His)/pCEP4 were passed through Ni-column (Novagen). The column eluents were fractionated by SDS-PAGE and TNFR-6 alpha-(His) was detected by western blot analysis using the anti-poly(His)<sub>6</sub> antibody (Sigma).

Production of HVEM/TR2-Fc, LT $\beta$ R-Fc and Flag-tagged soluble AIM-II (soluble AIM-II) fusion proteins were previously described (Zhai, Y., et al., *J. Clin. Invest.* 102, 1142-1151(1998)). Fc fusion protein-containing supernatants were filtered and trapped onto protein-G Sepharose beads. Flag-tagged soluble AIM-II proteins were purified with anti-Flag mAb affinity column.

*Immunoprecipitation.*

TNFR-6 alpha-(His) was incubated overnight with various Flag-tagged ligands of TNF superfamily and anti-Flag agarose in binding buffer (150 mM NaCl, 0.1% NP-40, 0.25% gelatin, 50 mM HEPES, pH 7.4) at 4°C, and then precipitated. The bound proteins were resolved by 12.5% SDS-PAGE and detected by western blot with HRP-conjugated anti-poly(His)<sub>6</sub> or anti-human IgG1 antibodies.

*Cell-binding assay.*

For cell-binding assays, HEK 293 EBNA cells were stably transfected using calcium phosphate method with pCEP4/full sequence of AIM-II cDNA or pCEP4 vector alone. After selection with Hygromycin B, cells were harvested with 1mM EDTA in PBS and incubated with TNFR-6 alpha-(His), HVEM/TR2-Fc, or LT $\beta$ R-Fc for 20 minutes on ice. For detecting Fc-fusion protein, cells were stained with FITC-conjugated goat anti-human IgG. To detect TNFR-6 alpha binding, cells were stained with anti-poly(His)<sub>6</sub> and FITC conjugated goat anti-mouse IgG consecutively. The cells were analyzed by FACScan (Becton Dickinson).

*Cytotoxicity Assay.*

Cytotoxicity assays using HT29 cells were carried out as described previously (Browning, J. L., et al., *J. Exp. Med.* 183, 867-878 (1996)). Briefly, 5000 HT29 cells were seeded in 96-well plates with 1% FBS, DMEM and treated with soluble AIM-II (10 ng/ml) and 10 units/ml human recombinant interferon- $\gamma$  (IFN- $\gamma$ ). Serial dilutions of TNFR-6 alpha-(His) were added in quadruplicate to microtiter wells. Cells treated with IFN- $\gamma$  and soluble AIM-II were incubated with various amounts of TNFR-6 alpha-(His) for 4 days before the addition of [ $^3$ H]thymidine for the last 6 h of culture. Cells were harvested, and thymidine incorporation was determined using a liquid scintillation counter.

**Results and Discussion***TNFR-6alpha is a new member of the TNFR superfamily*

TNFR-6 alpha was identified by searching an EST database. Three clones containing an identical open reading frame were identified from cDNA libraries of human normal prostate and pancreas tumor. A full-length TNFR-6 alpha cDNA encoding an intact N-terminal signal peptide was obtained from a human normal prostate library. The open reading frame of TNFR-6 alpha

encodes 300 amino acids. To determine the N-terminal amino acid sequence of mature TNFR-6 alpha, hexa-histidine tagged TNFR-6 alpha was expressed in mammalian cell expression system and the N-terminal amino acid sequence were determined by peptide sequencing. The N-terminal sequence of the processed mature TNFR-6 alpha-(His) started from amino acid 30, indicating that the first 29 amino acids constituted the signal sequence. Therefore, the mature protein of TNFR-6 alpha was composed of 271 amino acids with no transmembrane region. There was one potential N-linked glycosylation site (Asn173) in TNFR-6 alpha. Like OPG (Simmonet, W. *et al.*, *Cell* 89, 309-319 (1997)), the predicted protein was a soluble, secreted protein and the recombinant TNFR-6 alpha expressed in mammalian cells was ~40 kD as estimated on polyacrylamide gel. Alignment of the amino sequences of TNFR-I, TNFR-II, 4-1BB, TR2/HVEM, LT $\beta$ R, TR1/OPG and TNFR-6 alpha illustrated the existence of a potential cysteine-rich motif. TNFR-6 alpha contained two perfect and two imperfect cysteine-rich motifs and its amino acid sequence was remarkably similar to TR1/OPG amino acid sequence. TNFR-6 alpha shares ~30% sequence homology with OPG and TNFR-II.

#### *mRNA expression*

We analyzed expression of TNFR-6 alpha mRNA in human multiple tissues by Northern blot hybridization. Northern blot analyses indicated that TNFR-6 alpha mRNA was ~1.3 kb in length and was expressed predominantly in lung tissue and colorectal adenocarcinoma cell line SW480. RT-PCR analyses were performed to determine the expression patterns of TNFR-6 alpha in various cell lines. TNFR-6 alpha transcript was detected weakly in most hematopoietic cell lines. The expression of TNFR-6 alpha was induced upon activation in Jurkat T leukemia cells. Interestingly, TNFR-6 alpha mRNA was constitutively expressed in endothelial cell line, HUVEC at high level.

*Identification of the ligand for TNFR-6alpha*

To identify the ligand for TNFR-6 alpha, several Flag-tagged soluble proteins of TNF ligand family members were screened for binding to recombinant TNFR-6 alpha-(His) protein by immuno-precipitation. TNFR-6 alpha-(His) selectively bound AIM-II-Flag and FasL-Flag among Flag-tagged soluble TNF ligand members tested. This result indicates that TNFR-6 alpha binds at least two ligands, AIM-II and FasL. AIM-II exhibits significant sequence homology with the C-terminal receptor-binding domain of FasL (31%) but soluble AIM-II is unable to bind to Fas (Mauri, D. N., *et al.*, *Immunity* 8, 21-30 (1998); Zhai, Y., *et al.*, *J. Clin. Invest.* 102, 1142-1151 (1998)). They may have a similar binding epitope for TNFR-6 alpha binding.

Previously, Zhai and Harrop (Zhai, Y., *et al.*, *J. Clin. Invest.* 102, 1142-1151 (1998); Harrop, J. A., *et al.*, *J. Biol. Chem.* 273, 27548-27556 (1998)) reported the biological functions of AIM-II and its possible mechanisms of action as a ligand for HVEM/TR2 and/or LT $\beta$ R. AIM-II is expressed in activated T cells. AIM-II, in conjunction with serum starvation or addition of IFN- $\gamma$ , inhibits the cell proliferation in tumor cells, MDA-MB-231 and HT29.

To determine whether TNFR-6 alpha might act as an inhibitor to AIM-II interactions with HVEM/TR2 or LT $\beta$ R, TNFR-6 alpha-(His) was used as a competitive inhibitor in AIM-II-HVEM/TR2 interaction. When AIM-II was immunoprecipitated with HVEM/TR2-Fc in the presence of TNFR-6 alpha-(His), HVEM/TR2-Fc binding to AIM-II was decreased competitively by TNFR-6 alpha-(His) but TNFR-6 alpha-(His) binding to AIM-II was not changed by HVEM/TR2-Fc. Furthermore, the binding of HVEM/TR2-Fc (6 nM) or LT $\beta$ R (6 nM) was completely inhibited by 20 nM of TNFR-6 alpha-(His) protein in immunoprecipitation assays. These results support the notion that TNFR-6 alpha may act as a strong inhibitor of AIM-II function through

HVEM/TR2 and LT $\beta$ R.

*Binding of TNFR-6alpha-(His) to AIM-II-transfected cells*

To determine whether TNFR-6 alpha binds to AIM-II expressed on cell surface, we performed binding assay using AIM-II-transfected HEK 293 EBNA cells by flow cytometry. AIM-II-transfected HEK 293 EBNA cells were stained significantly by TNFR-6 alpha-(His) as well as by HVEM/TR2-Fc and LT $\beta$ R-Fc. No binding was detected by HVEM/TR2-Fc or LT $\beta$ R-Fc on pCEP4 vector-transfected HEK 293 EBNA cells. Furthermore, control isotype did not bind to AIM-II-transfected HEK 293 EBNA cells, and any of above fusion proteins did not bind to vector-transfected cells, confirming the specificity of these bindings. These bindings indicate that TNFR-6 alpha can bind to both soluble and membrane-bound forms of AIM-II.

*TNFR-6alpha inhibits AIM-II-induced cytotoxicity in HT29 cells*

Browning *et al.* (*J. Exp. Med.* 183, 867-878 (1996)) have shown that Fas activation leads to rapid cell death (12-24h) whereas LT $\beta$ R takes 2-3 days in induction of apoptosis for colorectal adenocarcinoma cell line, HT29. Zhai *et al.* (*J. Clin. Invest.* 102, 1142-1151 (1998)) also reported that AIM-II leads to the death of the cells expressing both LT $\beta$ R and HVEM/TR2 but not the cells expressing only the LT $\beta$ R or HVEM/TR2 receptor. Both HVEM/TR2 and LT $\beta$ R are involved cooperatively in AIM-II-mediated killing of HT29 cells (Zhai, Y., *et al.*, *J. Clin. Invest.* 102, 1142-1151(1998)).

To determine whether binding of TNFR-6 alpha inhibits AIM-II-mediated cytotoxicity, HT29 cells were incubated with 10 ng/ml of soluble AIM-II and IFN- $\gamma$  (10 U/ml) in the presence of 200 ng/ml of LT $\beta$ R-Fc or TNFR-6 alpha-(His). TNFR-6 alpha-(His) blocked significantly the AIM-II-mediated cell killing. Cells were also incubated with soluble AIM-II and/or IFN- $\gamma$  in the presence of varying concentration of TNFR-6 alpha-(His).

TNFR-6 alpha-(His) blocked soluble AIM-II-induced cell death in a dose-

dependent manner. Taken together, TNFR-6 alpha appears to act as a natural inhibitor of AIM-II-induced tumor cell killing. The data also suggest that TNFR-6 alpha contributes to immune evasion of tumors.

AIM-II interaction with HVEM/TR2 and/or LT $\beta$ R may trigger the distinct biological events, such as T cell proliferation, blocking of HVEM-dependent HSV1 infection and anti-tumor activity (Mauri, D. N., *et al.*, *Immunity* 8, 21-30 (1998); Zhai, Y., *et al.*, *J. Clin. Invest.* 102, 1142-1151 (1998); Harrop, J. A., *et al.*, *J. Biol. Chem.* 273, 27548-27556 (1998)).

TNFR-6 alpha may act as an inhibitor of AIM-II interaction and may play diverse roles in different cell types. TNFR-6 alpha may act as a decoy receptor and contribute to immune evasion both in slow and rapid tumor cell death, that are mediated by AIM-II and/or FasL mediated apoptosis pathway.

Another possibility is that TNFR-6 alpha may function as a cytokine to trigger membrane-bound FasL or AIM-II and directly transduce signals through FasL or AIM-II. Recently Desbarats and Suzuki groups reported that FasL could itself transduce signals, leading to cell-cycle arrest and cell death in CD4 $^{+}$  T cells but cell proliferation in CD8 $^{+}$  T cells (Desbarats, J., *et al.*, *Nature medicine* 4, 1377-1382 (1998); Suzuki, I., *et al.*, *J. Exp. Med.* 187, 123-128 (1998)). Therefore, TNFR-6 alpha may be involved in signaling through FasL and AIM-II.

HUVEC cells constitutively expressed TNFR-6 alpha in RT-PCR analysis. AIM-II and FasL have been known to be expressed in activated T cells. Therefore it is speculated that TNFR-6 alpha and its ligands are important for interactions between activated T lymphocytes and endothelium. TNFR-6 alpha may be involved in activated T cell trafficking as well as endothelial cell survival.

***Example 8: Activation-induced Apoptosis Assay***

Activation-induced apoptosis is assayed using SupT-13 T leukemia cells and is measured by cell cycle analysis. The assay is performed as follows. SupT-13 cells are maintained in RPMI containing 10% FCS in logarithmic growth (about  $1 \times 10^6$ ). Sup-T13 cells are seeded in wells of a 24 well plate at  $0.5 \times 10^6$ /ml, 1 ml/well. AIM II protein or Fas Ligand protein (0.01, 0.1, 1, 10, 100, 1000 ng/ml) or buffer control is added to the wells and the cells are incubated at 37°C for 24 hours in the presence or absence of the TNFR polypeptides of the invention. The wells of another 24 well plate are prepared with or without anti-CD3 antibody by incubating purified BC3 mAb at a concentration of 10 µg/ml in sterile-filtered 0.05M bicarbonate buffer, pH 9.5 or buffer alone in wells at 0.5 ml/well. The plate is incubated at 4°C overnight. The wells of antibody coated plates are washed 3 times with sterile PBS, at 4°C. The treated Sup-T13 cells are transferred to the antibody coated wells and incubated for 18 hrs., at 37°C. Apoptosis is measured by cell cycle analysis using propidium iodide and flow cytometry. Proliferation of treated cells is measured by taking a total of 300 µl of each treatment well and delivering in to triplicate wells (100 µl/well) of 96 well plates. To each well add 20 µl/well  $^3$ H-thymidine (0.5 µCi/20 µl, 2 Ci/mM) and incubate 18 hr., at 37°C. Harvest and count  $^3$ H-thymidine uptake by the cells. This measurement may be used to confirm an effect on apoptosis if observed by other methods. The positive controls for the assay is Anti-CD3 crosslinking alone, Fas Ligand alone, and/or AIM-II alone. In addition, profound and reproducible apoptosis in this line using anti-Fas monoclonal antibody (500 ng/ml in soluble form-IgM mAb) has been demonstrated. The negative control for the assay is medium or buffer alone. Also, crosslinking with another anti-CD3 mAB (OKT3) has been shown to have no effect. TNFR agonists according to the invention will demonstrate a reduced apoptosis when

compared to the treatment of the Sup-T13 cells with AIM-II or Fas Ligand in the absence of the TNFR agonist. TNFR antagonists of the invention can be identified by combining TNFR polypeptides having Fas Ligand or AIM-II binding affinity (e.g., mature TNFR) with the TNFR polypeptide to be tested and contacting this combination in solution with AIM-II or Fas Ligand and the Sup-T13 cells. The negative control for this assay is a mixture containing the mature TNFR, Sup-T13 cells, and AIM-II or Fas Ligand (FasL) alone. Samples containing TNFR antagonists of the invention will demonstrate increased apoptosis when compared to the negative control.

If an effect is observed by cell cycle analysis the cells can be further stained for the TUNEL assay for flow cytometry or with Annexin V, using techniques well known to those skilled in the art.

*Example 9: Blocking of Fas ligand Mediated Apoptosis of Jurkat T-cells by TNFR6 alpha-Fc*

*Methods.*

Jurkat T-cells which express the Fas receptor were treated either with sFas ligand alone or with sFas ligand in combination with Fas-Fc, or TNFR6 alpha-Fc (corresponding to the full length TNFR 6 alpha protein (amino acids 1-300 of SEQ ID NO:2) fused to an Fc domain, as described herein). The sFas ligand protein utilized was obtained from Alexis Corporation and contains a FLAG epitope tag at its N-terminus. As it has been demonstrated previously that cross-linking of Fas ligand utilizing the monoclonal Flag epitope enhances significantly the ability of Fas ligand to mediate apoptosis, the Flag antibody was included in this study. Specifically, 106 Jurkat cells (RPMI + 5%serum) were treated with Fas ligand (Alexis) (20ng/ml) and anti-Flag Mab (200ng/ml) and then incubated at 37°C for 16 hrs. When TNFR6 alpha -Fc was included in

the assay, the receptor was preincubated with the Fas ligand and anti-Flag Mab for 15 mins.

*Results*

After incubation, cells were harvested, resuspended in PBS and subjected to Flow Cytometric Analyses (Table V). In the absence of Fas ligand (FasL), approximately 1% of cells appear to be undergoing apoptosis as measured by high annexin staining and poor propidium iodide staining (Table IV). Treatment with soluble Fas ligand alone resulted in an approximate 7-fold increase in the number of apoptotic cells which as expected could be blocked in the presence of Fas-Fc. Similar to Fas-Fc, TNFR6 alpha -Fc was also capable of blocking Fas mediated apoptosis with the blocking by TNFR6 alpha-Fc observed in a dose dependent manner over three logarithmic scales (Table V). The ability of TNFR6 alpha -Fc to block Fas mediated killing of Jurkat cells was also determined in a cell death assay (Figures 7A-B). In this assay, cells were again treated with combinations of Fas ligand and TNFR6 alpha-Fc for 16 hrs. To measure the levels of viable cells after treatment, cells were incubated for 5 hrs with 10% ALOMAR blue and examined spectrophotometrically at OD 570nm-630nm. Treatment with Fas ligand resulted in a 50% decrease in cell viability (Figures 7A-B). The decrease in cell viability can be overcome by either Fas-Fc or TNFR6 alpha -Fc but not TR5-Fc (Figures 7A-B), confirming the ability of TNFR6 alpha to interfere with Fas ligand mediated activity. The ability of TNFR6 alpha -Fc at both 100 ng/ml and at 10 ng/ml to block Fas ligand mediated activity in this assay is statistically different ( $p < 0.05$ ) than when no TNFR6 alpha -Fc is added (Figures 7A-B). Furthermore, the ability of TNFR6 alpha -Fc to block Fas ligand mediated cell death and apoptosis appears to be as efficient with Fas-Fc (Table V and Figures 7A-B).

**Table V. FACS Analysis revealing blocking of Fas ligand mediated apoptosis:**  $10^6$  Jurkat cells (RPMI + 5% serum) were treated with Fas ligand (Allexis; 20 ng/ml) and anti-FLAG (200 ng/ml) and then incubated at 37° C for 16 hours. When Fc receptor was included in the assay, the receptor was preincubated with the Fas ligand and anti-FLAG Mab for 15 minutes. After incubation, cells were harvested, resuspended in PBS and subjected to Flow Cytometric Analyses.

	<i>Treatment</i>	<i>% Cells undergoing apoptosis</i>
10	Control (buffer)	1.24
	FasL (20 ng)	8.87
	FasL (20 ng)+ Fas-Fc (100 ng)	1.78
	FasL (20 ng)+ TNFR6 alpha-Fc (100 ng)	1.24
15	FasL (20 ng)+ TNFR6 alpha-Fc (10 ng)	2.79
	FasL (20 ng)+ TNFR6 alpha-Fc (1 ng)	7.95
	FasL (20 ng)+ TNFR6 alpha -Fc (0.1 ng)	8.58

*Conclusions.*

TNFR6 alpha-Fc appears to block Fas ligand mediated apoptosis of Jurkat cells in a dose dependent manner as effectively as Fas ligand.

**Example 10: Protein Fusions of TNFR-6 alpha and/or TNFR-6 beta**

TNFR-6 alpha and/or TNFR-6 beta polypeptides of the invention are optionally fused to other proteins. These fusion proteins can be used for a variety of applications. For example, fusion of TNFR-6 alpha and/or TNFR-6 beta polypeptides to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See EP A 394,827; Traunecker, *et al.*, *Nature* 331:84-86 (1988).) Similarly, fusion to IgG-1, IgG-3, and albumin increases the halflife time *in vivo*. Nuclear localization signals fused to TNFR-6 alpha and/or TNFR-6 beta polypeptides can target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase

or decrease the activity of a fusion protein. Fusion proteins can also create chimeric molecules having more than one function. Finally, fusion proteins can increase solubility and/or stability of the fused protein compared to the non-fused protein. All of the types of fusion proteins described above can be made using techniques known in the art or by using or routinely modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule.

Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also preferably contain convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian expression vector.

For example, if the pC4 (Accession No. 209646) expression vector is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and TNFR-6 alpha and/or TNFR-6 beta polynucleotide, isolated by the PCR protocol described in Example 1, is ligated into this BamHI site. Note that the polynucleotide is cloned without a stop codon, otherwise a fusion protein will not be produced.

If the naturally occurring signal sequence is used to produce the secreted protein, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., International application publication number WO 96/34891.)

Human IgG Fc region:

GGGATCCGGAGCCCCAAATCTTCTGACAAAACTCACACATGCCAACGTGCCAGCACCTGAATTGAGGGTGCAC  
CGTCAGTCCTCCCTCTCCCCCAAAACCAAGGACACCCCTCATGATCTCCCGACTCCTGAGGTCACATGCGTGG  
TGGTGGACGTAAGCCACGAAGACCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCA

5 AGACAAAGCCGGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCTCACCGTCCTGCACCAGGACT  
GGCTGAATGGCAAGGAGTACAAGTGCAGGTCTCCAACAAAGCCCTCCAAACCCCATCGAGAAAACCATCTCCA  
AAGCCAAAGGGCAGCCCCGAGAACACAGGTGTACACCCCTGCCCTGCCATGCCGGATGAGCTGACCAAGAACCAAGG  
TCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAAGCGACATGCCGTGGAGTGGGAGAGCAATGGGCAGCCGG  
AGAACAACTACAAGACCACGCCCTCCCCTGGACTCCGACGGCTCTTCTTCTACAGCAAGCTCACCGTGG  
ACAAGAGCAGGTGGCAGCAGGGAACGTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACACACGC  
AGAAGAGCCTCCCTGTCTCCGGTAAATGAGTGCGACGGCCGACTCTAGAGGAT  
(SEQ ID NO:27)

10 *Example 11: Production of an Antibody*

a) Hybridoma Technology

15 The antibodies of the present invention can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, cells expressing TR6-alpha and/or TR6-beta are administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of TR6-alpha and/or TR6-beta protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

20 Monoclonal antibodies specific for protein TR6-alpha and/or TR6-beta are prepared using hybridoma technology. (Kohler et al., Nature 256:495 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, an animal (preferably a mouse) is immunized with TR6-alpha and/or TR6-beta polypeptide or, more preferably, with a secreted TR6-alpha and/or TR6-beta polypeptide-expressing cell. Such polypeptide-expressing cells are cultured in any suitable tissue culture medium, preferably in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56°C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 µg/ml of streptomycin.

The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (*Gastroenterology* 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the TR6-alpha and/or TR6-beta polypeptide.

Alternatively, additional antibodies capable of binding to TR6-alpha and/or TR6-beta polypeptide can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the TR6-alpha and/or TR6-beta protein-specific antibody can be blocked by TR6-alpha and/or TR6-beta. Such antibodies comprise anti-idiotypic antibodies to the TR6-alpha and/or TR6-beta protein-specific antibody and are used to immunize an animal to induce formation of further TR6-alpha and/or TR6-beta protein-specific antibodies.

For *in vivo* use of antibodies in humans, an antibody is "humanized". Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric and humanized antibodies are known in the art and are discussed infra. (See, for review, Morrison, *Science* 229:1202 (1985); Oi et al., *BioTechniques* 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO

8601533; Robinson et al., WO 8702671; Boulianne et al., Nature 312:643 (1984);  
Neuberger et al., Nature 314:268 (1985).)

5                   b)     Isolation Of Antibody Fragments Directed Against TR6-alpha  
and/or TR6-beta From A Library Of scFvs

Naturally occurring V-genes isolated from human PBLs are constructed into a library of antibody fragments which contain reactivities against TR6-alpha and/or TR6-beta to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein by reference in its entirety).

10                  Rescue of the Library. A library of scFvs is constructed from the RNA of human PBLs as described in PCT publication WO 92/01047. To rescue phage displaying antibody fragments, approximately 109 E. coli harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 µg/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to inoculate 50 ml of 2xTY-AMP-GLU, 2 x 108 TU of delta gene 3 helper (M13 delta gene III, see PCT publication WO 92/01047) are added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 µg/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in PCT publication WO 92/01047.

15                  M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harboring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37° C without shaking and then for a further hour at 37°C with shaking. Cells are spun down (IEC-Centra 8,400 r.p.m. for 10 min), resuspended in 300 ml 2xTY broth containing 100 µg ampicillin/ml

and 25 µg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37°C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 µm filter (Minisart NML; Sartorius) to give a final concentration of approximately 1013 transducing units/ml (ampicillin-resistant clones).

Panning of the Library. Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 µg/ml or 10 µg/ml of a polypeptide of the present invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37°C and then washed 3 times in PBS. Approximately 1013 TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37°C. The E. coli are then plated on TYE plates containing 1% glucose and 100 µg/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of Binders. Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of the polypeptide of the present invention in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized

by PCR fingerprinting (see, e.g., PCT publication WO 92/01047) and then by sequencing.

5           ***Example 12: Method of Determining Alterations in the TNFR-6 alpha and/or TNFR-6 beta Gene***

RNA is isolated from entire families or individual patients presenting with a phenotype of interest (such as a disease). cDNA is then generated from these RNA samples using protocols known in the art. (See, Sambrook.) The cDNA is then used as a template for PCR, employing primers surrounding regions of interest in SEQ ID NO:1. Suggested PCR conditions consist of 35 cycles at 95° C for 30 seconds; 60-120 seconds at 52-58° C; and 60-120 seconds at 70° C, using buffer solutions described in Sidransky, D., *et al.*, *Science* 252:706 (1991).

10           PCR products are then sequenced using primers labeled at their 5' end with T4 polynucleotide kinase, employing SequiT<sup>TM</sup> Polymerase. (Epicentre Technologies). The intron-exon borders of selected exons of TNFR-6 alpha and/or TNFR-6 beta are also determined and genomic PCR products analyzed to confirm the results. PCR products harboring suspected mutations in TNFR-6 alpha and/or TNFR-6 beta are then cloned and sequenced to validate the results of the direct sequencing.

15           PCR products of TNFR-6 alpha and/or TNFR-6 beta are cloned into T-tailed vectors as described in Holton, T.A. and Graham, M.W., *Nucleic Acids Research*, 19:1156 (1991) and sequenced with T7 polymerase (United States Biochemical). Affected individuals are identified by mutations in TNFR-6 alpha and/or TNFR-6 beta not present in unaffected individuals.

20           Genomic rearrangements are also observed as a method of determining alterations in the TNFR-6 alpha and/or TNFR-6 beta gene. Genomic clones isolated using techniques known in the art are nick-translated with digoxigenin-deoxy-uridine 5'-triphosphate (Boehringer Manheim), and FISH

performed as described in Johnson, *et al.*, *Methods Cell Biol.* 35:73-99 (1991). Hybridization with the labeled probe is carried out using a vast excess of human cot-1 DNA for specific hybridization to the TNFR-6 alpha and/or TNFR-6 beta genomic locus.

5           Chromosomes are counterstained with 4,6-diamino-2-phenylidole and propidium iodide, producing a combination of C- and R-bands. Aligned images for precise mapping are obtained using a triple-band filter set (Chroma Technology, Brattleboro, VT) in combination with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and variable excitation wavelength filters. (Johnson, *et al.*, *Genet. Anal. Tech. Appl.*, 8:75 (1991).) Image collection, analysis and chromosomal fractional length measurements are performed using the ISee Graphical Program System. (Inovision Corporation, Durham, NC.) Chromosome alterations of the genomic region of TNFR-6 alpha and/or TNFR-6 beta (hybridized by the probe) are identified as insertions, deletions, and translocations. These TNFR-6 alpha and/or TNFR-6 beta alterations are used as a diagnostic marker for an associated disease.

10

***Example 13: Method of Detecting Abnormal Levels of TNFR-6 alpha and/or TNFR-6 beta in a Biological Sample***

15           TNFR-6 alpha and/or TNFR-6 beta polypeptides can be detected in a biological sample, and if an increased or decreased level of TNFR-6 alpha and/or TNFR-6 beta is detected, this polypeptide is a marker for a particular phenotype. Methods of detection are numerous, and thus, it is understood that one skilled in the art can modify the following assay to fit their particular needs.

20           For example, antibody-sandwich ELISAs are used to detect TNFR-6 alpha and/or TNFR-6 beta in a sample, preferably a biological sample. Wells of a microtiter plate are coated with specific antibodies to TNFR-6 alpha and/or TNFR-6 beta, at a final concentration of 0.2 to 10 ug/ml. The

antibodies are either monoclonal or polyclonal and are produced using technique known in the art. The wells are blocked so that non-specific binding of TNFR-6 alpha and/or TNFR-6 beta to the well is reduced.

5       The coated wells are then incubated for > 2 hours at RT with a sample containing TNFR-6 alpha and/or TNFR-6 beta. Preferably, serial dilutions of the sample should be used to validate results. The plates are then washed three times with deionized or distilled water to remove unbounded TNFR-6 alpha and/or TNFR-6 beta.

10      Next, 50 ul of specific antibody-alkaline phosphatase conjugate, at a concentration of 25-400 ng, is added and incubated for 2 hours at room temperature. The plates are again washed three times with deionized or distilled water to remove unbounded conjugate.

15      75 ul of 4-methylumbelliferyl phosphate (MUP) or p-nitrophenyl phosphate (NPP) substrate solution is then added to each well and incubated 1 hour at room temperature to allow cleavage of the substrate and fluorescence. The fluorescence is measured by a microtiter plate reader. A standard curve is prepared using the experimental results from serial dilutions of a control sample with the sample concentration plotted on the X-axis (log scale) and fluorescence or absorbance on the Y-axis (linear scale). The TNFR-6 alpha and/or TNFR-6 beta polypeptide concentration in a sample is then 20     interpolated using the standard curve based on the measured fluorescence of that sample.

25      *Example 14: Method of Treating Decreased Levels of TNFR-6 alpha and/or TNFR-6 beta*

The present invention relates to a method for treating an individual in need of a decreased level of TNFR-6 alpha and/or TNFR-6 beta biological activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of TNFR-6 alpha

and/or TNFR-6 beta antagonist. Preferred antagonists for use in the present invention are TNFR-6 alpha and/or TNFR-6 beta-specific antibodies.

Moreover, it will be appreciated that conditions caused by a decrease in the standard or normal expression level of TNFR-6 alpha and/or TNFR-6 beta in an individual can be treated by administering TNFR-6 alpha and/or TNFR-6 beta, preferably in a soluble and/or secreted form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of TNFR-6 alpha and/or TNFR-6 beta polypeptide comprising administering to such an individual a pharmaceutical composition comprising an amount of TNFR-6 alpha and/or TNFR-6 beta to increase the biological activity level of TNFR-6 alpha and/or TNFR-6 beta in such an individual.

For example, a patient with decreased levels of TNFR-6 alpha and/or TNFR-6 beta polypeptide receives a daily dose 0.1-100 ug/kg of the polypeptide for six consecutive days. Preferably, the polypeptide is in a soluble and/or secreted form.

***Example 15: Method of Treating Increased Levels of TNFR-6 alpha and/or TNFR-6 beta***

The present invention also relates to a method for treating an individual in need of an increased level of TNFR-6 alpha and/or TNFR-6 beta biological activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of TNFR-6 alpha and/or TNFR-6 beta or an agonist thereof.

Antisense technology is used to inhibit production of TNFR-6 alpha and/or TNFR-6 beta. This technology is one example of a method of decreasing levels of TNFR-6 alpha and/or TNFR-6 beta polypeptide, preferably a soluble and/or secreted form, due to a variety of etiologies, such as cancer.

For example, a patient diagnosed with abnormally increased levels of

TNFR-6 alpha and/or TNFR-6 beta is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the is determined to be well tolerated.

5

***Example 16: Method of Treatment Using Gene Therapy - Ex Vivo***

One method of gene therapy transplants fibroblasts, which are capable of expressing soluble and/or mature TNFR-6 alpha and/or TNFR-6 beta polypeptides, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. *et al.*, *DNA*, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding TNFR-6 alpha and/or TNFR-6 beta can be amplified using PCR primers which correspond to the 5' and 3' end encoding sequences respectively. Preferably, the 5' primer contains an EcoRI site and

the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments.

5 The ligation mixture is then used to transform *E. coli* HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector contains properly inserted TNFR-6 alpha and/or TNFR-6 beta.

10 The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the TNFR-6 alpha and/or TNFR-6 beta gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the TNFR-6 alpha and/or TNFR-6 beta gene (the packaging cells are now referred to as producer 15 cells).

20 Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector 25 that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether TNFR-6 alpha and/or TNFR-6 beta protein is produced.

The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier

beads.

***Example 17: Method of Treatment Using Gene Therapy - In Vivo***

Another aspect of the present invention is using *in vivo* gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) TNFR-6 alpha and/or TNFR-6 beta sequences into an animal to increase or decrease the expression of the TNFR-6 alpha and/or TNFR-6 beta polypeptide. The TNFR-6 alpha and/or TNFR-6 beta polynucleotide may be operatively linked to a promoter or any other genetic elements necessary for the expression of the TNFR-6 alpha and/or TNFR-6 beta polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, International Application publication number WO90/11092, International Application publication number WO98/11779; US Patent NO. 5693622, 5705151, 5580859; Tabata H. *et al.*, *Cardiovasc. Res.* 35:470-479 (1997); Chao J. *et al.*, *Pharmacol. Res.* 35:517-522 (1997); Wolff J.A. *Neuromuscul. Disord.* 7:314-318 (1997); Schwartz B. *et al.*, *Gene Ther.* 3:405-411 (1996); Tsurumi Y. *et al.*, *Circulation* 94:3281-3290 (1996) (incorporated herein by reference).

The TNFR-6 alpha and/or TNFR-6 beta polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The TNFR-6 alpha and/or TNFR-6 beta polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the

TNFR-6 alpha and/or TNFR-6 beta polynucleotides may also be delivered in liposome formulations (such as those taught in Felgner P.L. *et al.*(1995) Ann. NY Acad. Sci. 772:126-139 and Abdallah B. *et al.*(1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

5           The TNFR-6 alpha and/or TNFR-6 beta polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

10           The TNFR-6 alpha and/or TNFR-6 beta polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue.

15           Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be

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achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked TNFR-6 alpha and/or TNFR-6 beta polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked TNFR-6 alpha and/or TNFR-6 beta polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected TNFR-6 alpha and/or TNFR-6 beta polynucleotide in muscle *in vivo* is determined as follows. Suitable TNFR-6 alpha and/or TNFR-6 beta template DNA for production of mRNA coding for TNFR-6 alpha and/or TNFR-6 beta polypeptide is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is

made on the anterior thigh, and the quadriceps muscle is directly visualized. The TNFR-6 alpha and/or TNFR-6 beta template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for TNFR-6 alpha and/or TNFR-6 beta protein expression. A time course for TNFR-6 alpha and/or TNFR-6 beta protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of TNFR-6 alpha and/or TNFR-6 beta DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be used to extrapolate proper dosages and other treatment parameters in humans and other animals using TNFR-6 alpha and/or TNFR-6 beta naked DNA.

**Example 18: Rescue of Ischemia in Rabbit Lower Limb Model**

To study the in vivo effects of TNFR-6 alpha and/or TNFR-6 beta on ischemia, a rabbit hindlimb ischemia model is created by surgical removal of one femoral arteries as described previously (Takeshita, S. et al., *Am J. Pathol* 147:1649-1660 (1995)). The excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels originating from the internal iliac artery (Takeshita, et al., *Am J. Pathol* 147:1649-1660 (1995)). An interval of 10 days is allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. At 10

day post-operatively (day 0), after performing a baseline angiogram, the internal iliac artery of the ischemic limb is transfected with 500 mg naked TNFR-6 alpha and/or TNFR-6 beta expression plasmid by arterial gene transfer technology using a hydrogel-coated balloon catheter as described (Riessen, R. et al., *Hum Gene Ther.* 4:749-758 (1993); Leclerc, G. et al., *J. Clin. Invest.* 90: 936-944 (1992)). When used in the treatment, a single bolus of 500 mg protein or control is delivered into the internal iliac artery of the ischemic limb over a period of 1 min. through an infusion catheter. On day 30, various parameters are measured in these rabbits: (a) BP ratio - The blood pressure ratio of systolic pressure of the ischemic limb to that of normal limb; (b) Blood Flow and Flow Reserve - Resting FL: the blood flow during undilated condition and Max FL: the blood flow during fully dilated condition (also an indirect measure of the blood vessel amount) and Flow Reserve is reflected by the ratio of max FL: resting FL; (c) Angiographic Score - This is measured by the angiogram of collateral vessels. A score is determined by the percentage of circles in an overlaying grid that with crossing opacified arteries divided by the total number in the rabbit thigh; (d) Capillary density - The number of collateral capillaries determined in light microscopic sections taken from hindlimbs.

The studies described in this example test activity in TNFR-6 proteins. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of TNFR-6 alpha and/or TNFR-6 beta.

**Example 19: Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models**

**A. Diabetic db+/db+ Mouse Model.**

To demonstrate that TNFR-6 accelerates the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness

wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al.*, *J. Surg. Res.* 52:389 (1992); Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)).

The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/+m) littermates. Mutant diabetic (db+/db+) mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al.* *Proc. Natl. Acad. Sci. USA* 77:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al.*, *J. Immunol.* 120:1375 (1978); Debray-Sachs, M. *et al.*, *Clin. Exp. Immunol.* 51(1):1-7 (1983); Leiter *et al.*, *Am. J. of Pathol.* 114:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular lesions, basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al.*, *Exp. Neurol.* 83(2):221-232 (1984); Robertson *et al.*, *Diabetes* 29(1):60-67 (1980); Giacomelli *et al.*, *Lab Invest.* 40(4):460-473 (1979); Coleman, D.L., *Diabetes* 31 (Suppl):1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al.*, *J. Immunol.* 120:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this model may be similar to the healing observed in human diabetes (Greenhalgh, *et al.*, *Am. J. of Pathol.* 136:1235-1246 (1990)).

Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and were 8 weeks old at the beginning of the study. Animals are individually housed and

received food and water *ad libitum*. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

5           Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med.* 172:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

10           Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

15           TNFR-6 alpha and/or TNFR-6 beta is administered using at a range different doses of TNFR-6 protein, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

20           Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then

harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

5 Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are evaluated: 1) Vehicle placebo control, 2) TNFR-6 alpha and/or TNFR-6 beta.

10 Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 was 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations were made using the following formula:

$$[ \text{Open area on day 8} ] - [ \text{Open area on day 1} ] / [ \text{Open area on day 1} ]$$

15 Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of 20 the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with TNFR-6. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 25 (1990)). A calibrated lens micrometer is used by a blinded observer.

Tissue sections are also stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by

evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

#### ***B. Steroid Impaired Rat Model***

The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, S.M. Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahl, S.M. *et al.*, *J. Immunol.* 115: 476-481 (1975); Werb, Z. *et al.*, *J. Exp. Med.* 147:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert, R.H., *et al.*, *An. Intern. Med.* 37:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991); Haynes, B.F. *et al.*, *J. Clin. Invest.* 61: 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M., "Glucocorticoids and wound healing", In: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991);

Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M., "Glucocorticoids and wound healing", In: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989); Pierce, G.F. *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 2229-2233 (1989)).

5 To demonstrate that TNFR-6 alpha and/or TNFR-6 beta can accelerate the healing process, the effects of multiple topical applications of TNFR-6 on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

10 Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and were 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

15 The wounding protocol is followed according to section A, above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue was no longer visible and the wound is covered by a continuous epithelium.

TNFR-6 alpha and/or TNFR-6 beta is administered using a range different doses of TNFR-6 protein, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Four groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) were evaluated: 1) Untreated group 2) Vehicle placebo control 3) TNFR-6 treated groups.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 was 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations were made using the following formula:

$$\frac{[\text{Open area on day 8}] - [\text{Open area on day 1}]}{[\text{Open area on day 1}]}$$

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining was

performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin was improved by treatment with TNFR-6 alpha and/or TNFR-6 beta. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

5 Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

10 The studies described in this example test activity in TNFR-6 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of TNFR-6 alpha and/or TNFR-6 beta.

#### ***Example 20: Lymphadema Animal Model***

15 The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and histopathology. Acute lymphedema is observed for 20 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks.

25 Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue.

Circumference and volumetric measurements are then made following injection of dye into paws.

Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

Using a microscope, muscles in back of the leg (near the semitendinosus and adductors) are bluntly dissected. The popliteal lymph node is then located.

The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then and ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous peak, which typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw are measured before operation and daily for 7 days. The effect plasma proteins on lymphedema is determined and whether protein analysis is a useful testing parameter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

Circumference Measurements: Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people and the readings are averaged. Readings are taken from both control and edematous limbs.

Volumetric Measurements: On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

Blood-plasma protein measurements: Blood is drawn, spun, and serum separated prior to surgery and the conclusion to the experiment to measure for total protein and Ca<sup>2+</sup> comparison.

Limb Weight Comparison: After drawing blood, the animal is prepared for tissue collection. The limbs were amputated using a quillotine, then both experimental and control legs were cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint is disarticulated and the foot is weighed.

Histological Preparations: The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at – 80 degree C until sectioning. Upon sectioning, the muscle was observed under fluorescent microscopy for lymphatics. Other immuno/histological methods are currently being evaluated.

The studies described in this example test activity in TNFR-6 proteins. However, one skilled in the art could easily modify the exemplified studies to

test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of TNFR-6 alpha and/or TNFR-6 beta.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

**Example 21: TNFR6-Fc Inhibits FasL mediated toxicity in a ConA Mouse Model of Liver Injury**

The intravenous administration of Concanavalin A to mice activates T lymphocytes and induces both apoptotic and necrotic cell death of hepatocytes, mimicking aspects of the pathophysiology of chronic active hepatitis (Tiegs et al., J. Clin. Invest. 90: 196. (1992)). Fas-Fc protein, a dimeric form of Fas expected to inhibit Fas ligand activity, has been reported to reduce liver injury in this model via inhibition of Fas ligand demonstrating an involvement of Fas pathway in the pathology (Ksontini et al., J Immunol.; 60(8):4082-4089 (1998)).

*Validation of model:*

To validate the ConA mouse model, Con A was administered intravenously to Balb/c mice at 10, 15, and 20 mg/kg dose of ConA along with placebo or Fas-Fc at 97.5 micrograms/mouse. 10 Balb/c mice were used per treatment group. The mice were sacrificed 22 hours after treatment, serum collected and biochemical analysis performed using a Clinical Chemistry Analyzer ILAB900 (Instrumentation Laboratory) to determine the levels of the liver specific transaminases, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are released in the serum upon liver damage ((Tiegs et al., J. Clin. Invest. 90: 196. (1992)). The administration of FasFc at a dose of 97.5 micrograms/mouse (about 5 mg/kg) was found to significantly inhibit the elevated liver enzymes at ConA doses of 10 and 15 mg/kg but not at 20 mg/kg (data not shown), thus validating the model.

Balb/C mice were injected intravenously with ConA (15 mg/kg) together with or without a three log dose of TNFR6-Fc (0.6, 6. 60 ug/mouse). The TNFR6-Fc fusion protein used in this example corresponds to the full length TNFR6-alpha polypeptide sequence (amino acids 1-300 of SEQ IDNO:2) fused to an Fc domain. 10 Balb/c mice were used per treatment group. The mice were sacrificed 22 hours after treatment and serum levels of ALT and AST were determined using a Clinical Chemistry Analyzer ILAB900 (Instrumentation Laboratory). The administration of TNFR6-Fc significantly inhibited both ALT and AST levels at the highest dose tested (60 micrograms/mouse, 3 mg/kg) by 50% (data not shown). Thus TNFR6-Fc significantly reduced ConA induced serum AST and ALT in a dose response fashion.

*Effect of TNFR6-Fc on ConA induced apoptotic events in the liver*

Since the elevation in serum liver enzyme levels reflects both apoptotic and non-apoptotic pathways of hepatocyte destruction, a more critical determination of the extent of liver injury can be derived via direct

measurement of apoptotic events. Thus apoptosis was analyzed using whole liver cell suspensions isolated from mice treated with TNFR6-Fc and Con A. Three independent markers of apoptosis were assessed on the same sample. These include changes in surface expression of phosphatidylserine, measurements of DNA damage, and caspase activation.

Balb/C mice were injected intravenously with ConA (15 mg/kg) together with or without a three log dose of TNFR6-Fc (0.6, 6. 60 ug/mouse). Cell suspensions were isolated from the livers of 3 mice/group and liver cells were isolated by placing the intact liver tissue on a 70 µm cell strainer and teased apart with the stopper of a 5cc syringe using RPMI 1640/10% FBS. To remove red blood cells and large piece of tissue debris, the filtered cell suspension was layered over lymphocyte separation medium (density 1.0770 g/ml). The interface layer was collected, washed and the cells were counted. Prior to FACS analysis, the cell suspension was refiltered over a 40 µm filter.

For measurement of Annexin V binding (an indicator of apoptosis), cells were first incubated with fluorochrome-conjugated monoclonal antibodies CD45 CyChrome and B220 or anti-TCR $\beta$  PE (Pharmingen, San Diego, CA). Cells were washed with binding buffer (Pharmingen) then incubated with Annexin V FITC (Pharmingen). Stained cells were acquired and analyzed using a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA). Only CD45 positive events were collected. Cells staining brightly for B220 and Annexin V were considered apoptotic B cells; cells staining brightly for anti-TCR $\beta$  and Annexin V were considered apoptotic T cells.

The level of DNA degradation (another hallmark of apoptosis) was determined by Terminal UTP nick-end labeling (TUNEL) which measures this degradation by using TdT enzyme to add FITC-labeled dUTP to the 3' ends of nicked DNA using the Apo-DIRECT kit (Pharmingen) according to manufacturer's directions. Briefly, cells were fixed in 1% paraformaldehyde,

washed in PBS and then fixed with ice-cold 70% ethanol. Cells were washed twice in washing buffer, then incubated with staining solution containing TdT enzyme and dUTP-FITC at 37°C for one hour. Cells were washed twice with rinsing buffer, re-suspended in propidium iodide solution and acquired on the FACScan. For analysis, an electronic gate was set on singlet events, and cells staining brightly for dUTP-FITC were considered apoptotic cells.

To determine the presence of the active form of caspase-3 (an early indicator of apoptosis) cells were incubated in IC FIX (BioSource International, Camarillo, CA), washed twice in PBS, then permeabilized with IC PERM (BioSource). Cells were incubated with 5 µg rabbit anti-caspase-3 PE (Pharmingen) in IC PERM, washed in IC PERM, then washed twice with PBS. Cells were acquired on the FACScan and analyzed for PE mean fluorescence.

For all three indicators of apoptosis, TNFR6-Fc inhibited apoptosis in livers of mice as compared to mice treated with Con A alone (Table VI). Using DNA damage as a marker and TUNEL analysis, a dose-dependent trend of inhibition with TR6-Fc was observed. These data support a role for TNFR6-Fc in inhibition of apoptosis in ConA-induced hepatitis.

**Table VI.** Apoptosis of liver cells isolated from TNFR6-Fc-treated mice.<sup>1</sup>

Treatment	Percent % Apoptotic Cells measured by:			
	Tunel	Caspase-3	Annexin V/TcRβ	Annexin V/B220
Untreated Control	-	18.7	2.0	6.1
Con A (15 mg/kg) Control	24.4	35.2	7.0	12.2
TNFR6-Fc (0.6 µg/mouse)	15.6	22.7	3.5	6.3
TNFR6-Fc (6.0 µg/mouse)	13.6	22.5	2.3	2.9
TNFR6-Fc (60 µg/mouse)	9.5	20.3	3.0	4.2

<sup>1</sup>Liver cell suspensions were analyzed for apoptosis using one of three independent measures. DNA degradation was measured using TUNEL staining; caspase activation by the analysis of the active form of caspase-3; and annexin V staining of surface membrane changes. Cell suspensions were isolated from the livers of 3 mice/group and pooled. The resulting pooled suspension was used to perform each analysis. For Annexin-V staining, only liver CD45+ cells were acquired and Annexin-V staining assessed on cells costained for B220 or TcRβ.

#### **Conclusion:**

The findings that TNFR6-Fc reduced both ConA induced serum AST and ALT levels and ConA induced liver cell apoptosis supports the therapeutic application of TNFR6-alpha and TNFR6-beta polypeptides of the invention for the treatment and/or prevention of hepatitis and other

forms of liver injury.

5 applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

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